

The diverse effects of CD98 in terms of its structural/functional relationship

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DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous candidature for a higher degree. All work presented in this thesis was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

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ABSTRACT

CD98 constitutively and specifically associates with $\beta 1$ integrins and is highly expressed on the surface of tumour cells irrespective of tissue of origin. This investigation has demonstrated that over expression of the CD98 heavy chain promotes both anchorage-independent and serum-independent growth. This oncogenic activity is dependent on phosphoinositol-3-OH kinase stimulation and the level of surface expression of CD98. Using chimeras of the CD98 heavy chain and the Type II membrane protein CD69, I have shown that the transmembrane domain is necessary and sufficient for integrin association. Amino acids 82-87 in the putative cytoplasmic/transmembrane region appear to be critical for the oncogenic potential of CD98 and provide a novel mechanism for tumour promotion by integrins. This demonstrates how high expression of CD98 in human cancers causes transformation. The transmembrane association of CD98 and $\beta 1$ integrins may provide a unique target to intervene in cancer.

The diverse effects of CD98 prompted an investigation in to whether there were any other molecules like CD98 which had similar effects on integrins. The Neural cell adhesion molecule (NCAM), a membrane associated glycoprotein of the Ig superfamily appeared to be a potential candidate. This study has shown that CD98 and NCAM and NCAM and $\beta 1$ colocalise together and indicate that NCAM, CD98 and $\beta 1$ may form a complex inducing a signalling cascade.

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ABBREVIATIONS

3-PPI's	3-phosphorylated phosphoinositides
4F2	CD98 monoclonal antibody
α subunit	alpha subunit
AA	amino acids
ADP/ATP	Adenosine Diphosphate/Adenosine Triphosphate
ARF	ADP Ribosylation Factor
β subunit	beta subunit
BSA	Bovine Serum Albumin
CAM's	Cell Adhesion Molecules
CD	Cluster Designation molecules
CD47/IAP	Integrin Associated Protein
CD98HC	CD98 heavy chain
CD98LC	CD98 light chain
CEA-CAM	Carcinoembryonic Antigen-related Cell Adhesion Molecule
CHO's	Chinese Hamster Ovary cells
CIB	Calcium Binding Protein
CMF/PBS	Calcium/Magnesium Free PBS
DMEM	Dulbecco's Modified Eagles Medium
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol bis Tetraacetic Acid
ERK	Extracellular-signal-Regulated Kinases
FAC's	Fluorescence Activated Cell Sorter

F-actin	Filamentous actin
FAK	Focal Adhesion Kinase
FBGC's	Foreign Body Giant Cells
FBS/FCS	Foetal Bovine/Calf Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Iso Thio Cyanate
FKHR-1	Forkhead Family Transcription Factor-1
FSG	Fish Skin Gelatin
GAP	GTPase Activating Proteins
GDP/GTP	Guanosine 5'Diphosphate/Triphosphate
GPI-	Glycosylphosphatidylinositol
HA	Haemagglutinin
HGF	Hepatocyte Growth Factor
HIV-1	Human Immunodeficiency Virus-1
HLA	Histocompatibility Antigen
HN	Haemagglutinin Neuramidase
HRP	Horse Radish Peroxidase
ICAM-1	Intercellular Adhesion Molecule-1
ICAP-1	Integrin Cytoplasmic Domain Associated Protein -1
IGF-1	Insulin Growth Factor
IL-2	Interleukin 2
ILK	Integrin Linked Kinase
IPTG	Isopropyl-beta-d-thiogalactopyranoside
JNK	Jun-kinase
LAD	Leucocyte Adhesion Deficiency
LAT	L-type amino acid transporter
	Lymphocyte-Function Associated
LFA-1	Molecule-1
mAbs	monoclonal antibodies

Mac-1	$\alpha_M\beta_2$ (only found on macrophages)
MAdCAM	Mucosal Addressin Cell Adhesion Molecule-1
MAPK	Mitogen-Activated Protein Kinase
MbCD	Methyl β cyclodextrin
MEK	MAP kinase kinase
MgCl₂	Magnesium chloride
MLCK	Myosin Light Chain Kinase
MMP's	Matrix Metalloproteinases
MTT	3-2,5- diphenyl tetrazolium bromide
NaCl	Sodium chloride
NCAM	Neural Cell Adhesion Molecule
NCAM-Fc	Neural Cell Adhesion Molecule Fusion protein
NSCLC	Non-Small Cell Lung Cancer
p70 RSK	p70 Ribosomal S6 kinase
PAGE	Polyacrylamide Gel Electrophoresis
PAK	p21 Activated Kinase
PBMC's	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PECAM	Platlet Endothelial Cell Adhesion Molecule
PFA	Paraformaldehyde
PI3K	Phosphoinositol 3-OH kinase
PIP's	Phosphatidylinositol phosphates
PKB/AKT	Protein Kinase B
PKC	Protein Kinase C
PKCδ	Protein Kinase C δ
PLCγ	Phospholipase C γ
PSA	polsialylation

PSF	Point Spread Function
PTK	Protein Tyrosine Kinases
RANKL	Receptor Activator of NFkB Ligand
RPMI	Rosewell Park Memorial Institute Medium
RTK's	Receptor Tyrosine Kinases
SCF	Stem Cell Factor
SCLC	Small Cell Lung Cancer
SDS	Sodium dodecyl sulphate
SEM	standard error of the mean
TBS	Tris Buffered Saline
TLC	Thin Layer Chromatography
TM4SF	Transmembrane 4 Superfamily
TRAP	Tartrate Resistant Acid Phosphatase
TSP-1	Thrombospondin-1
uPAR	urokinase-type-Plasminogen Activator Receptor
UV	Ultra Violet
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor

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CHAPTER 1

INTRODUCTION

Normal cells and tissues exist in defined and regulated relationships to components of the extracellular matrix (ECM) in their immediate micro-environment (Keely et al., 1998). The life and death situation at the cellular level is therefore profoundly influenced by components of the ECM (Stupack and Cheresch, 2002), which mediate events such as tissue re-modelling, differentiation and development. Many of these processes are governed by members of a family of cell-surface adhesion receptors known as integrins. Integrins play critical roles in many aspects of cell physiology and when aberrations occur within these interactions pathological disorders can develop. Many cancers show abnormalities of integrin function and this may be as a consequence of transformed cells losing their appropriate contacts with the ECM. These transformed cells then acquire anchorage independence and a motile-invasive phenotype (Keely et al., 1998). Although much is known about the extracellular interactions between integrins and their ligands, significantly less is known about the biochemical pathways that integrins regulate and the cellular functions which are thereby controlled (Clark and Brugge, 1995).

1.1 INTEGRINS

Integrins are a large family of heterodimeric cell surface receptors that mediate attachment to the extracellular matrix and cell surface ligands (Fernandez et al., 1998). They are expressed on a wide variety of cells and most cells express several integrins. Integrins are receptors for ECM proteins such as fibronectin, laminin, vitronectin and collagen,(Table 1).

The phenomenon “anchorage dependence” was first described by Stoker et al., (1968). They found that normal cells were blocked in the G1 phase of the cell cycle

when cultured in suspension. This indicated a requirement of cell adhesion for growth in normal cells. The ability of integrins to bind to various ECM proteins mediates important cell-cell and cell-matrix adhesive interactions involved in many cellular functions. These include, embryogenesis, wound healing, platelet aggregation, programmed cell death, and leucocyte homing and activation. As a result of their involvement in cell adhesion, integrins are also implicated in tumour cell growth and metastasis. However in addition to their roles as adhesion receptors, integrins also play a role in signalling. They have been found to have a role in regulating reorganization of the cytoskeleton, intracellular ion transport, lipid metabolism, kinase activation and gene expression. This variety of functional roles is a consequence of integrins ability to regulate ligand binding. This regulation of ligand binding can occur in two different ways, either by a conformational change or by clustering many integrins together. This will be discussed in more detail in section 1.1.3 and 1.1.4.

<u>Ligand</u>	<u>Integrin</u>
Adenovirus penton base protein	$\alpha_5\beta_3, \alpha_5\beta_5$
Bone sialoprotein	$\alpha_5\beta_3, \alpha_5\beta_5$
<i>Borrelia burgdorferi</i>	$\alpha_{IIb}\beta_3$
<i>Candida albicans</i>	$\alpha_M\beta_2$
Collagens	$\alpha_1\beta_1, \alpha_2\beta_1, \alpha_{11b}\beta_1, \alpha_{1b}\beta_3$
Denatured collagen	$\alpha_5\beta_1, \alpha_5\beta_3, \alpha_{IIb}\beta_3$
Cytotactin/tenascin-C	$\alpha_8\beta_1, \alpha_9\beta_1, \alpha_5\beta_3, \alpha_5\beta_6$
Decorsin	$\alpha_{IIb}\beta_3$
Disintegrins	$\alpha_5\beta_3, \alpha_{IIb}\beta_3$
E Cadherin	$\alpha_E\beta_7$
Echovirus 1	$\alpha_2\beta_1$
Epiligrin	$\alpha_3\beta_1$
Factor X	$\alpha_M\beta_2$
Fibronectin	$\alpha_2\beta_1, \alpha_3\beta_1, \alpha_4\beta_1, \alpha_4\beta_7, \alpha_5\beta_1, \alpha_8\beta_1, \alpha_5\beta_1, \alpha_5\beta_3, \alpha_5\beta_5, \alpha_5\beta_6, \alpha_5\beta_8, \alpha_{IIb}\beta_3$
Fibrinogen	$\alpha_5\beta_1, \alpha_M\beta_2, \alpha_5\beta_3, \alpha_x\beta_2, \alpha_{IIb}\beta_3$
HIV Tat Protein	$\alpha_5\beta_3, \alpha_5\beta_5$
iC3b	$\alpha_M\beta_2, \alpha_x\beta_2$
ICAM-1	$\alpha_L\beta_2, \alpha_M\beta_2$
ICAM-2,3,4,5	$\alpha_L\beta_2$
Invasin	$\alpha_3\beta_1, \alpha_4\beta_1, \alpha_5\beta_1, \alpha_6\beta_1$
Laminin	$\alpha_1\beta_1, \alpha_2\beta_1, \alpha_6\beta_1, \alpha_7\beta_1, \alpha_6\beta_4, \alpha_5\beta_3$
MAdCAM-1	$\alpha_4\beta_7$
Matrix metalloproteinase-2	$\alpha_5\beta_3$
Neutrophil inhibitory factor	$\alpha_M\beta_2$
Osteopontin	$\alpha_5\beta_3$
Plasminogen	$\alpha_{IIb}\beta_3$
Prothrombin	$\alpha_5\beta_3, \alpha_{IIb}\beta_3$
Sperm fertilin	$\alpha_6\beta_1$
Thrombospondin	$\alpha_3\beta_1, \alpha_5\beta_3, \alpha_{IIb}\beta_3$
VCAM-1	$\alpha_4\beta_1, \alpha_4\beta_7$
Vitronectin	$\alpha_5\beta_1, \alpha_5\beta_3, \alpha_5\beta_5, \alpha_{IIb}\beta_3$
von Willebrand factor	$\alpha_5\beta_3, \alpha_{IIb}\beta_3$

Table 1. Integrin extracellular ligands

This table summarises the major extracellular ligands of integrins. This table also demonstrates the ability of individual integrin family members to recognize multiple ligands. Table adapted from Plow et al, 2000.

1.1.1 INTEGRIN STRUCTURE

Integrins are heterodimeric transmembrane receptors composed of non-covalently linked alpha and beta subunits. Each subunit has a type I transmembrane protein and a large N-terminus extracellular domain, a single membrane spanning region, and a short C-terminal cytoplasmic tail (Shattil et al., 1998). The integrin receptor family consists of at least 18 distinct alpha subunits and 8 beta subunits, leading to a large number of possible combinations (Juliano et al., 2002). Some of these integrin subunits are promiscuous, ie. $\beta 1$ interacts with lots of alpha subunits, (as shown in figure 1.1.1a), whereas some subunits only interact with one specific subunit. The diversity of integrins is expanded further by alternative splicing, post-translational modifications, and interactions with other cell-surface and intracellular molecules (Plow et al., 2000). The alpha subunits are approximately 1000 residues in length and the beta subunits are approximately 750 residues in length.

The specificity of an integrin binding to a particular ligand appears to depend primarily on the extracellular portion of the alpha subunit. However both subunits are required for an integrin complex to function properly. The binding of integrins to ligands depends on the extracellular divalent cations present, (Ca^{2+} , Mn^{2+} or Mg^{2+} , depending on the integrin). This reflects the presence of the 3-4 divalent cation-binding domains in the extracellular domain of the alpha subunit. The alpha subunits all contain seven tandem repeats of about 60 amino acids at their N-terminus. The last three or four of these repeats contain sequences (Asp-x-Asp-x-Asp-Gly-x-x-Asp) or related sequences that are thought to contribute to the divalent cation-binding properties of these subunits, (Hynes, 1992). Gailit and Ruoslahti., (1988) and Kirchhofer et al., (1990,1991) speculated that the nature of these cations could affect both affinity and specificity for ligands and that they were essential for alpha (α) beta (β) subunit associations of some integrins. The role of divalent cations in integrin function was demonstrated by lack of ligand binding upon removal of cations by chelating agents. Mn^{2+} , Mg^{2+} and Ca^{2+} were found to have distinct effects on integrin function *in vitro*. With $\beta 1$ integrins, Mn^{2+}

confers high affinity binding properties, whereas Ca^{2+} inhibits binding of ligand and Mg^{2+} stimulates ligand binding.

β subunits have a characteristic four fold repeat of a cysteine rich segment, which is believed to be internally disulphide bonded, (Calvete et al., 1991). This segment appears to contribute to the ligand binding domain. A diagram of the current proposed model can be seen in figure 1.1.1b.

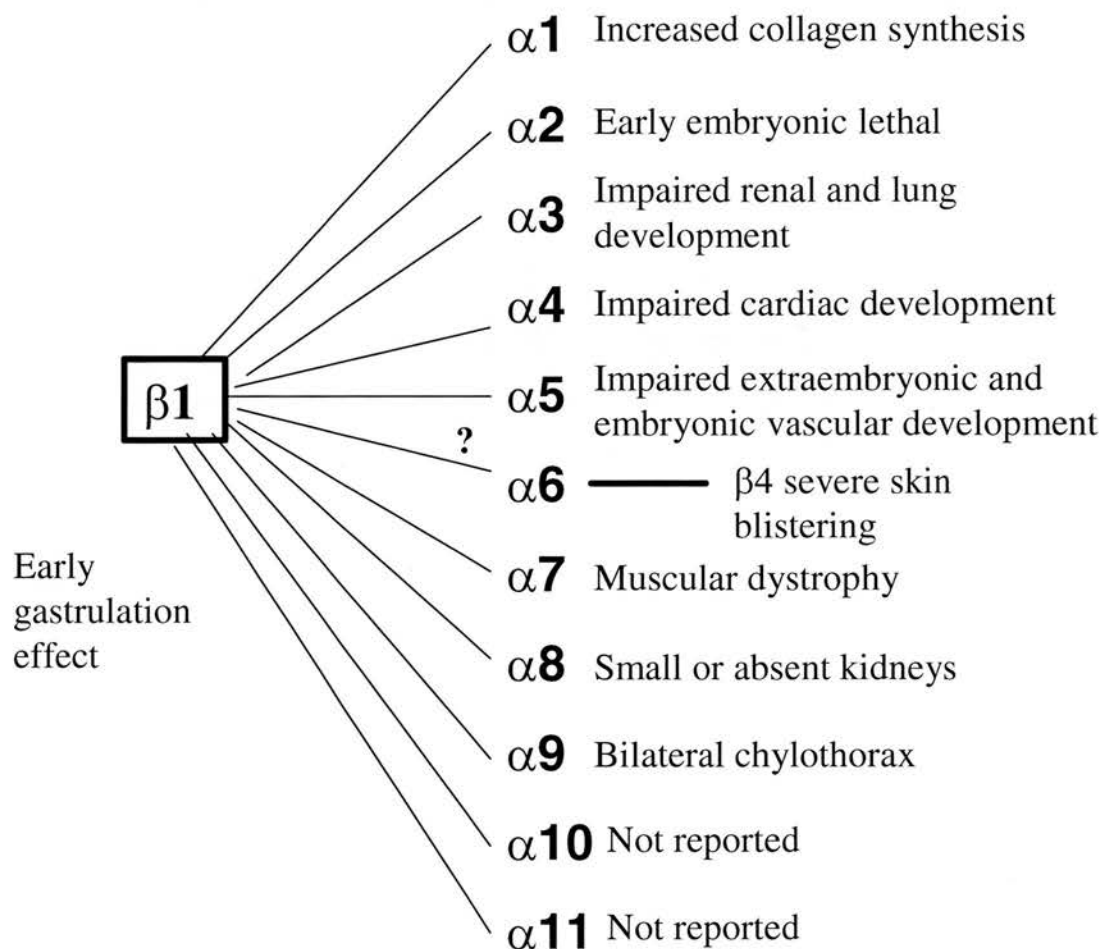


Figure 1.1.1a The $\beta 1$ subfamily

The largest subfamily of integrins is the group of heterodimers which share the $\beta 1$ subunit. Figure adapted from Sheppard, 2000.

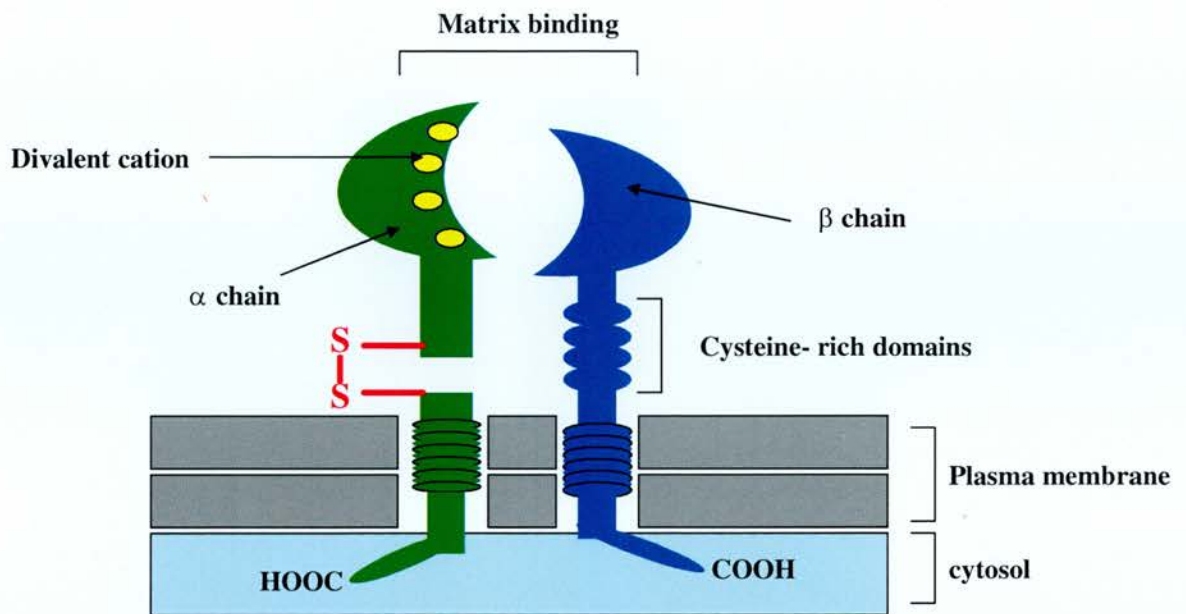


Figure 1.1.1b The subunit structure of an integrin

The figure above shows the structure of an integrin. An integrin is a heterodimeric cell surface receptor, composed of non-covalently linked α and β subunits. Ligand binding is dependent on the presence of divalent cations either Ca^{2+} or Mg^{2+} . Adapted from Alberts et al., Molecular Biology Of The Cell.

1.1.2 INTEGRIN SIGNALLING

During the ligand binding process integrins undergo dynamic changes, involving conformational changes and/or receptor clustering. Integrins are components of both “inside-out” and “outside-in” signalling mechanisms. Inside-out signalling is initiated by specific intracellular proteins interacting with the cytoplasmic tail of the integrin, stimulating a conformational change to the extracellular-ligand binding sites. Outside-in signalling occurs when integrin receptors engage and cluster together leading to the formation of focal adhesions, generating a cytoplasmic signalling cascade. However in some cases both inside-out and outside-in signalling play a role in transmitting signals in biological systems. One integrin which has been studied in great depth is the $\alpha_{IIb}\beta_3$, which is the predominant complex in platelets and is involved in haemostasis. Inside-out signalling leads to the conversion of $\alpha_{IIb}\beta_3$ from a low affinity/avidity state to a high affinity/avidity state. This determines whether $\alpha_{IIb}\beta_3$ can engage soluble ligands such as fibrinogen and consequently whether platelet aggregation occurs. Outside-in signalling is then involved in the post-ligand binding events. In terms of $\alpha_{IIb}\beta_3$, these events include platelet spreading, granule secretion and clot retraction (Shattil et al., 1998). $\alpha_{IIb}\beta_3$ has therefore been a powerful model to study integrin activation as discussed below, (figure 1.1.2).

Integrin affinity modulation

Integrin adhesive functions can itself be regulated by cellular signals. Integrin activation is often caused by intracellular signals which change the affinity of some integrins for their ligands, a process known as affinity modulation (Hughes and Pfaff., 1998). A classical example of integrin affinity modulation at work is platelet aggregation. On resting circulating platelets, the integrin $\alpha_{IIb}\beta_3$ does not bind to any soluble ligands. However when fibrinogen, a soluble plasma protein which binds to $\alpha_{IIb}\beta_3$ is crosslinked with platelets, platelet aggregation occurs. Platelet activation is

induced by various agonists such as thrombin, ADP and epinephrine which causes a conformational change to the $\alpha_{IIb}\beta_3$ integrin, increases its binding affinity for fibrinogen resulting in platelet activation (Shattil et al., 1998). A further conformational change occurs on ligand binding (Freilinger et al., 1988; 1990; 1991). A role for tyrosine kinases in integrin activation/inside-out signalling and platelet activation was suggested by Savage et al., (1996). They observed that tyrosine kinase inhibitors partially blocked fibrinogen binding and platelet aggregation. Another signalling molecule which has been implicated in integrin function is Phosphatidylinositol 3-OH Kinase (PI3Kinase) and its products, phosphatidylinositol phosphates (PIP's), PIP2 and PIP3. In platelets, the agonist thrombin stimulates an increase in PIP3 leading to an increase in PIP2. Inhibitors of PI3Kinase partially block agonist-induced activation of $\alpha_{IIb}\beta_3$ and platelet aggregation. It has been suggested that the 3-phosphorylated phosphoinositides function to stabilise binding to fibrinogen.

Another example of inside out signalling is an essential feature of leucocyte adhesion. Integrins such as $\alpha_4\beta_1$ and two β_2 integrins, $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1), found on leucocytes, mediate cell-cell adhesion and sometimes cell-substrate adhesion. The process of adhesion is a critical aspect of leucocyte function and is therefore tightly regulated. When leucocytes and platelets circulate in the blood stream, they need to remain non-adhesive. However at sites of inflammation they must be able to recognise and adhere to the endothelium and then migrate through the vessel wall. This regulation is very complex and therefore requires inside-out signalling for activation and deactivation of integrins (Brown and Hogg., 1996). In the genetic disease, leucocyte adhesion deficiency (LAD), β_2 integrins are absent and the leucocyte cannot adhere stably to the endothelium. This leads to recurrent pyogenic infections, problems with wound healing and the development of gingivitis (severe inflammation of the gums) in patients suffering from LAD.

Conformational changes may be modified by the divalent cations present in the alpha subunit. This role for divalent cations was suggested after studies using monoclonal antibodies (mAbs), which recognised conformation dependent epitopes in β_1 integrins. Stimulatory mAbs such as TS2/16, 8A2, A1A5 and 12G10, and inhibitory

antibodies such as mAb13, 4B4 and P4C10, all recognized a twelve amino acid sequence present in the β A domain.

The conformational changes required for ligand binding to $\alpha_{IIb}\beta_3$ could be regulated by intracellular molecules which bind to the cytoplasmic tails of the integrin or integrin associated membrane proteins. These will be described in more detail in section 1.1.6.

Integrin avidity modulation

Outside-in signalling or integrin avidity is the process in which integrins mediate information transfer into cells by integrin clustering and ligation. Outside-in signalling collaborates with signals originating from growth factor receptors and other plasma membrane receptors to regulate a host of anchorage-dependent cellular functions (Shattil et al., 1998).

As mentioned earlier, platelet functions are regulated by outside-in signalling, such as granule secretion and secondary aggregation. These outside-in signals determine the ultimate size of a hemostatic plug or a pathological thrombus and are initiated at localized regions of cell-matrix and cell-cell contact. Signals are triggered by oligomerization and clustering of $\alpha_{IIb}\beta_3$ in platelets, and then propagated by interactions between integrin cytoplasmic tails and signalling molecules and cytoskeletal proteins (Shatill et al., 1998).

An example of avidity modulation is the specific kinase FAK (focal adhesion kinase). FAK is a 125kDa non-receptor tyrosine kinase which localises to the plasma membrane at sites of integrin-mediated adhesion. A distinct domain of FAK directly associates with integrin cytoplasmic tails. A signalling cascade is initiated when integrins cluster as a consequence of an interaction with ECM proteins. This clustering creates a binding site for c-Src via integrin-associated FAK autophosphorylation of tyrosine 397. After c-Src has bound, additional tyrosines on FAK can become phosphorylated and in turn become docking sites for paxillin and grb2 and other signalling proteins, eventually leading to

the activation of Ras and MAP (mitogen-activated protein) kinase cascades (Brown and Hogg., 1996).

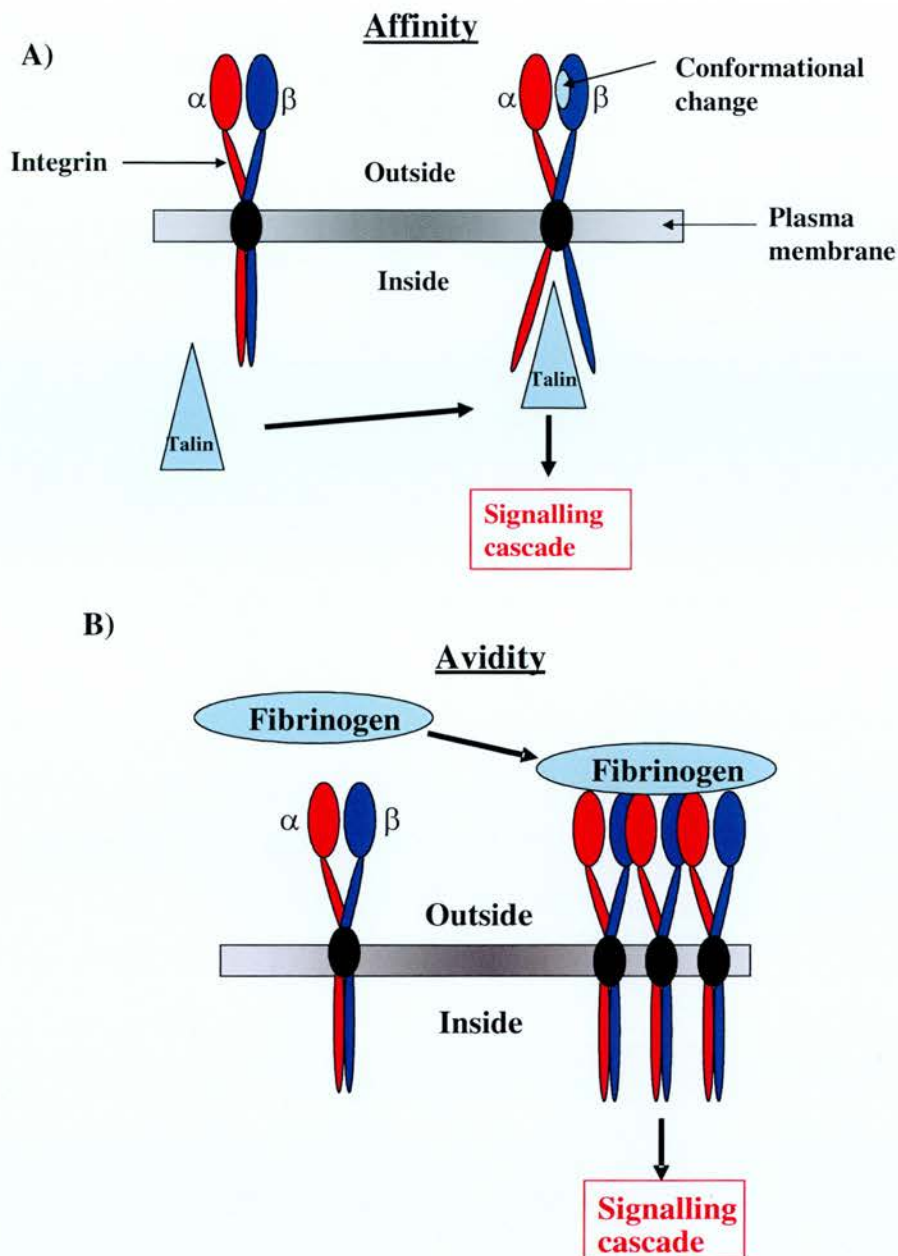


Figure 1.1.2 Integrin Affinity and Avidity modulation.

Panel A shows affinity modulation of integrin complexes which is initiated by interaction with specific intracellular proteins with the cytoplasmic tail of the integrin promoting a conformational change in the extracellular domain, resulting in increased affinity for ligands such as fibrinogen. Panel B represents avidity modulation which occurs when integrins cluster together at focal contacts promoting a cytoplasmic signal cascade.

1.1.3. INTEGRIN SIGNALLING PATHWAYS

In addition to mediation of cellular adhesion to ECM proteins, integrins also transduce intracellular signals that promote cell migration as well as cell survival and proliferation (Meredith et al., 1993; Stromblad and Cheresch, 1996). Unlike growth factor receptors, integrins, have no intrinsic enzymatic activity. They activate signalling pathways via the clustering of kinases and adaptor proteins in focal adhesion complexes (Jin and Varner, 2004). Signal transduction by integrins influence many growth regulatory pathways. Examples of this are the activation of tyrosine kinases such as FAK, pp60src and c-Abl; serine threonine kinases such as MAP kinases, jun kinase (JNK) and protein kinase C (PKC); intracellular ions such as protons (pH) and calcium; the small GTPase Rho and lipid mediators such as phosphoinositides, diacylglycerol and arachidonic acid metabolites (Shwartz et al., 1997).

Upon integrin stimulation, PKC and MAP kinase are activated, leading to cell attachment and spreading. An increase in intracellular calcium concentration can also be induced by integrin engagement and this calcium release is a key regulator of intracellular signalling via multiple integrins (Clark and Brugge, 1995). Phospholipid metabolites also have a role in integrin-mediated signal transduction, by activating PKC (Divecha and Irvine, 1995). Integrins also appear to regulate changes in gene expression, crucial for developmental and proliferative responses and integrins also affect cell survival, as mentioned earlier by regulating programmed cell death (Damsky and Werb, 1992; Yurochko et al., 1992; Schwartz and Ingber, 1994).

Focal Adhesion Kinase (FAK)

The tyrosine kinase FAK plays an important role in integrin-mediated signalling. A number of proteins tyrosines are phosphorylated upon integrin engagement, such as paxillin, tensin and FAK. The clustering of chimeric integrin receptors expressing several different β cytoplasmic domains is sufficient to induce FAK phosphorylation (Clark and Brugge, 1995). FAK is phosphorylated on at least 7 different tyrosines and

has the ability to interact with cellular proteins that contain SH2 domains such as Src, Grb2 and PI3-Kinase (Schlaepfer et al., 1994; Pawson, 1995; Brakenbusch et al., 2000). Through these interactions FAK has the capacity to integrate integrin-mediated signalling as shown in figure 1.1.3a.

After autophosphorylation of FAK on Tyr 397 a binding site is formed for Src. Once Src has bound, additional tyrosines on FAK can become phosphorylated and become a docking site for Grb2 (Brown and Hogg, 19996). Grb2 (growth factor receptor bound protein-2), links activated tyrosine kinase to an activator of mSOS1, a guanine nucleotide exchange factor that functions by converting inactive Ras-GDP to active Ras-GTP. This suggests that integrins may regulate the Ras/Raf/Mek/Erk pathway (Clark and Brugge, 1995).

FAK also binds to PI3-Kinase, which is thought to promote cell activation. Autophosphorylation of FAK on Tyr 397 also allows FAK to bind to and activate PI3-Kinase. PI3K influences integrin affinity and avidity but also contributes to activation of Rac-1 (Brakenbusch et al., 2002). Rac-1 is involved in membrane ruffling but also when activated stimulates p21 activated kinase (PAK) which then stimulates migration by increasing the turnover of focal adhesions (Kiosses et al., 1999). Increased levels of FAK expression have also been correlated with the invasiveness and metastatic phenotype in solid tumours (Weiner et al., 1993; Akasaka et al., 1995; Owens et al., 1995).

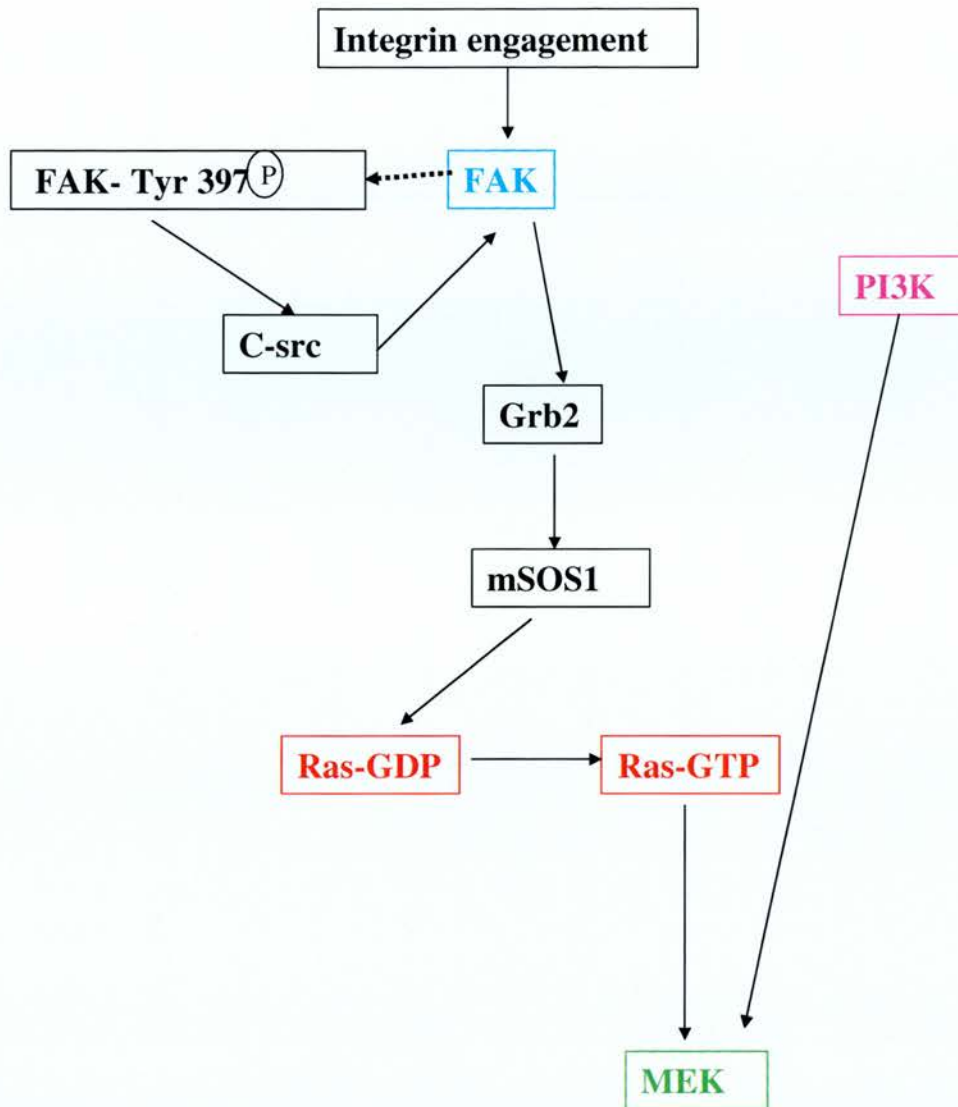


Figure 1.1.3a FAK signalling pathways

After integrin engagement, FAK is autophosphorylated on Tyr 397, forming a binding site for c-Src. Once c-Src is bound additional tyrosines on FAK are phosphorylated and form a docking site for Grb2. Grb2 once bound links FAK to mSOS1. mSOS1 converts inactive Ras-GDP to active Ras -GTP and this stimulates the Ras/Mek pathway. Autophosphorylation of FAK also allows FAK to bind and activate PI3-Kinase.

RAS-MAP Kinase pathway

Many groups have indicated a link between outside-in signalling through integrins and the activation of the Ras/MAPK pathways. Miyamoto et al., (1995), found that 20 signal transduction molecules, including Rho A, Rac-1, Ras, Raf, MAPK, MEK and JNK were colocalised with clustered integrins, suggesting they might be localised in focal adhesions. Ras is a member of the Ras-superfamily of small GTPases. As previously mentioned, the association of Grb2 and mSOS1 with FAK suggests that integrins may regulate Ras. Another mechanism of activation of the Ras/MAPK pathway does not involve FAK, but does involve caveolin-1, Fyn and the adaptor protein Shc. Integrin α subunits activate Fyn, causing tyrosine phosphorylation of Shc leading to recruitment of the Grb2/SOS complex. This triggers Ras and the downstream kinase cascade leading to Erk activation (Juliano, 2002) as shown in figure 1.1.3b.

Ras is activated by guanine nucleotide exchange factors, (GEF's) that release GDP and allow GTP binding. Ras-GTP then interacts with several effector proteins such as Raf and PI3-Kinase. When Raf is activated it then activates MAPK, Erk and Mek 1 + 2 (Hancock, 2003). MAP kinases have the requirement for both a tyrosine and a threonine to be phosphorylated for full activation. Erk phosphorylates tyrosine 185 and threonine 183 and this translocates Erk to the nucleus where it phosphorylates ELK-1. ELK-1 is a gene regulatory protein which is in a complex with the serum response factor, which upon phosphorylation binds to a specific DNA sequence (the serum response element) (Marais et al., 1993; Price et al., 1996). This then activates ELK-1 turning on the transcription of fos and then the transcription of cyclin D and p21cip, the former of which is involved in the induction of cell proliferation and the latter in degradation of the cyclin which facilitates passage through G1 and allows entry into the S-phase (Woods et al., 1997). Erk also phosphorylates MLCK (Myosin light chain kinase) which in turn phosphorylates Myosin light chain. This leads to a fully activational actin-myosin motor unit, serving the process of motility (Klemke et al., 1997).

There has also been a lot of focus on other members of the Ras superfamily, Rho, Rac and cdc42, (Parise et al., 2000). By activating Rho and Rac, cell migration is stimulated and actin filaments are anchored to the cell membrane (Ren et al., 1999). This in turn promotes cell cycle entry by stimulating cyclin expression (Assoian and Schwartz, 2001; Jin and Varner, 2004). The Rho family of small GTPases, therefore seems essential for the regulation of the actin cytoskeleton. Rho A has been implicated in the formation of stress fibres, while Rac and cdc42 regulate the cortical actin structures such as lamellipodia and filipodia (Parise et al., 2000). These findings indicate that there is a detailed pattern of communication between integrins, small GTPases and the actin cytoskeleton. This communication therefore plays an important role in functions which are characteristic of metastatic tumour cells, such as invasion and mobility.

Integrin engagement has been a suggested mechanism of activating members of Rho GTPases. A direct demonstration of this is a study where ligation of $\alpha_6\beta_4$ integrin in clone A colon carcinoma cells, activated Rho A (O'Connor et al., 2000). It is also thought that activated Rac and cdc42 couple to a variety of downstream effectors and this may be achieved by integrin engagement. However the evidence for this remains unclear (Parise et al., 2000).

In addition to a role in outside-in integrin signalling, small GTPases also play a role in inside-out signalling by converting integrins to a high affinity state. A constitutively active form of R-Ras (G38V) has been shown to increase adherence to fibronectin and an increase in binding of soluble fibronectin. Zhang et al., (1996), showed using CHO cells co-expressing both the platelet integrin $\alpha_{IIb}\beta_3$ and R-Ras (G38V), that $\alpha_{IIb}\beta_3$ was converted to a high affinity state as detected by a monoclonal antibody specific for the active conformation of the integrin. Hughes et al., (1997) showed that H-Ras and its downstream effector kinase Raf 1 suppresses integrin activation whereas Sethi et al., (1999) showed that R-Ras can reverse the suppressive effect of both activated H-ras and its effector Raf-, therefore regulating integrin affinity.

Another small GTPase which has been implicated as an integrin activator is Rap-1. Katagiri et al, (2000), found that Rap-1 is a potent activator of the leucocyte integrin

LFA-1 and Reedquist et al., (2000), found that Rap-1 is an important component of a pathway leading from CD31 or PECAM engagement to integrin activation.

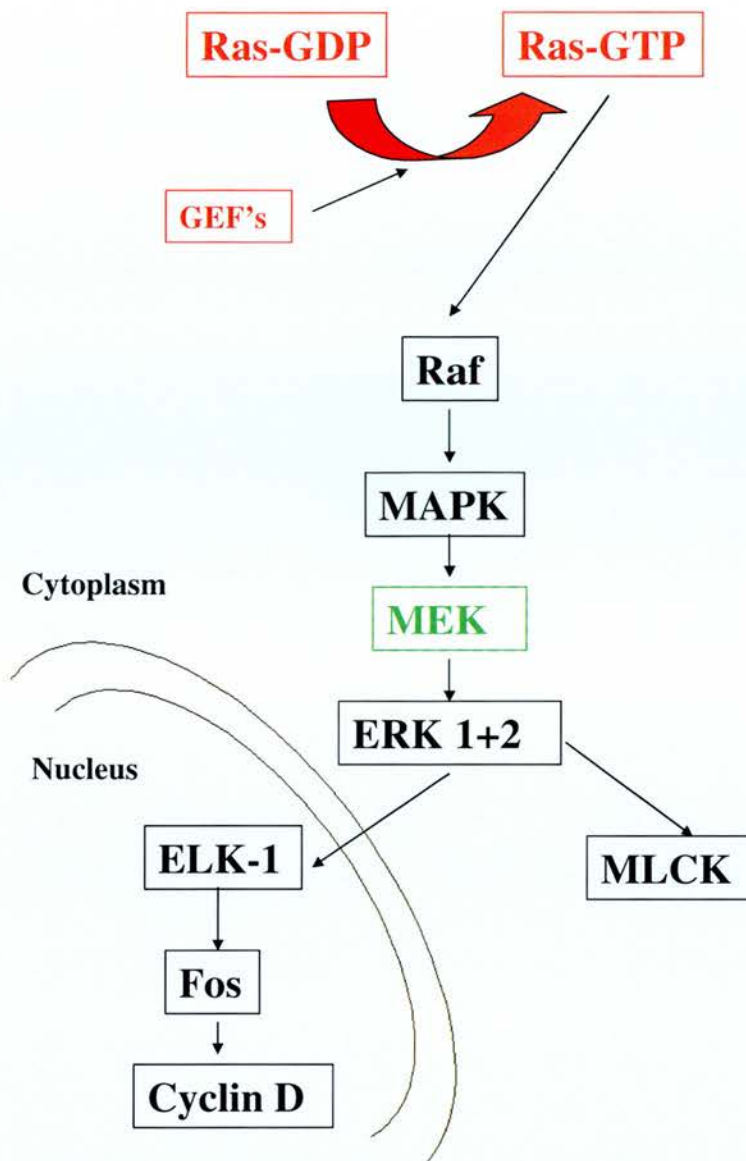


Figure 1.1.3b Ras/Mek pathway

Ras is activated by GEF's which release GDP and allow GTP binding. Ras-GTP then interacts with Raf and PI3-Kinase. Raf activates MAPK which activates MEK which in turn activates ERK 1+2. ERK phosphorylates tyrosine 185 and threonine 183 and this translocates ERK to the nucleus. In the nucleus ERK phosphorylates ELK-1 which turns on the transcription factor Fos which in turn transcribes cyclin D and many other genes and induces cell proliferation. Erk also phosphorylates MLCK (Myosin light chain kinase) which in turn phosphorylates Myosin light chain leading to a fully activational actin-myosin motor unit, serving the process of motility.

PI3 Kinase (Phosphoinositol 3 Kinase) -pathway

Promotion of cell survival can occur via MAPK (ERK), NF κ B, Bcl-2, PI3K and AKT, whereas proteins such as c-Jun kinase (JNK), p38-kinase and Bad and the caspase family of proteases can stimulate apoptosis. PI3-Kinase and its downstream effector PKB/AKT (a serine threonine kinase), often plays a role in the regulation of cell survival. After integrin engagement, receptor tyrosine kinases are activated and PI3-Kinase is recruited to phosphotyrosine residues. PI3-Kinase catalyses the phosphorylation of inositol lipids at the D-3 position of the inositol ring, resulting in the formation of 3-phosphorylated phosphoinositides (King et al., 1997). These products PtdIns(3,4,5)P₃ (PIP3) and PtdIns (3,4)P₂ (PIP2) then stimulate 3-phosphoinositide dependent protein kinase 1 and 2 (PDK1+2). These kinases phosphorylate Thr308 and Ser473 on AKT respectively. AKT then phosphorylates many different downstream substrates such as Bad (death effector) or phosphorylates Caspase-9, inhibiting its protease activity, leading to cell survival (Parise et al., 2000). This pathway can be seen more clearly in figure 1.1.3c.

As discussed earlier PI3-Kinase can be activated through autophosphorylation of FAK, which consequently activates Rac-1. In addition to its role in membrane ruffling, Rac-1 also activates PAK (p21 activated kinase) which phosphorylates Raf and Mek, therefore having effects on transcription factors and cell migration. PI3-Kinase also downregulates p21 cip, contributing to the induction of cyclin D1, thereby affecting cell cycle progression (Brakenbusch et al., 2002).

As mentioned earlier when normal cells are deprived of integrin-mediated anchorage to the ECM they undergo a form of apoptosis, "anoikis". The importance of FAK/ PI3K/ AKT pathway in anoikis has been reported by Frisch et al., (1996) and Khwaja et al., (1997), however it also appears that this pathway is important in cell survival and therefore an important mechanism to study in terms of malignancy. Reduced apoptosis can contribute to tumourgenesis, since it helps tumour cells to escape natural elimination (Brakenbusch et al., 2000). Many human tumours carry mutations in

the proteins of the PI3-kinase signalling pathway. This indicates the importance of PI3Kinase in the prevention of malignant proliferation (Brakenbusch et al., 2002).

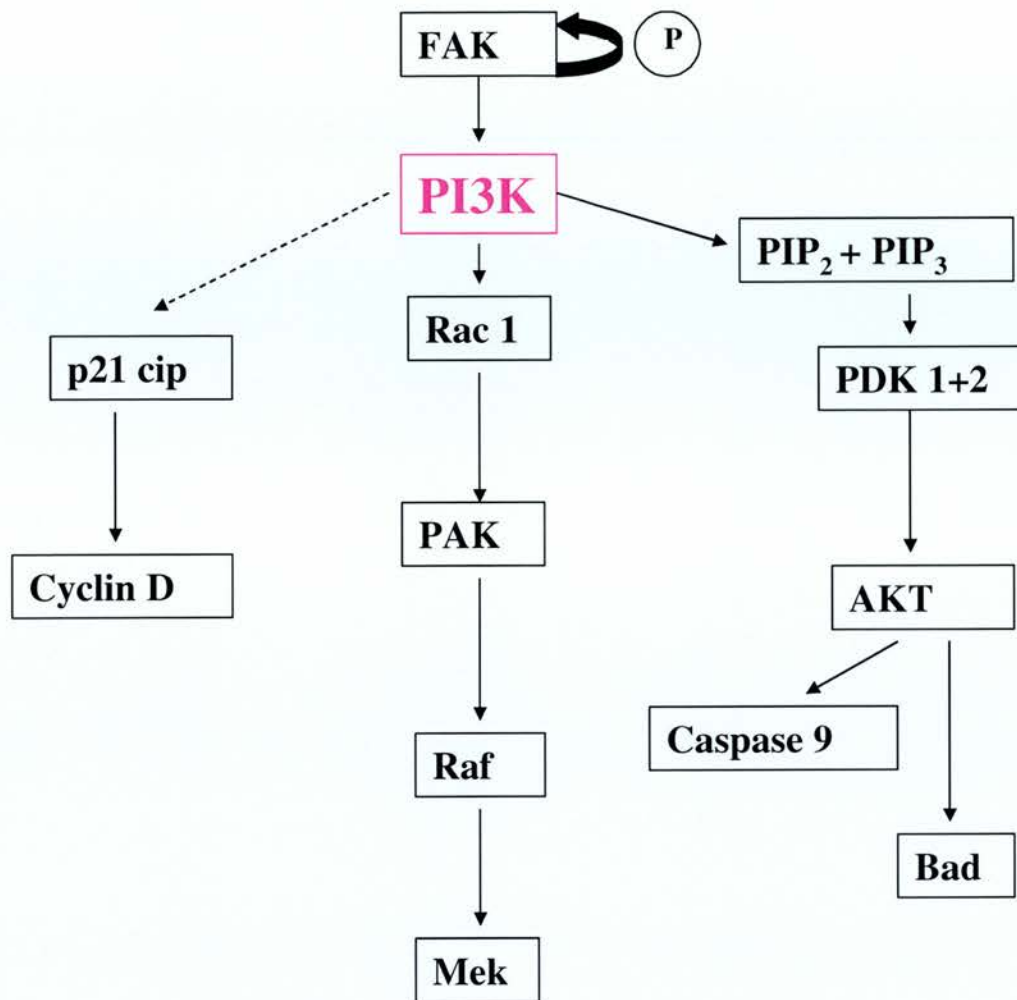


Figure 1.1.3c PI3-Kinase pathway

Through autophosphorylation of FAK, PI3-Kinase is activated. PI3-Kinase can then activate Rac-1, activating PAK which then phosphorylates Raf and MEK. PI3-Kinase can also downregulate p21cip inducing cyclin D which affects the cell cycle. PI3-Kinase can also promote cell survival through the production of AKT. The products PIP₃ and PIP₂, these then stimulate production of PDK 1+2 which in turn phosphorylates AKT. AKT then phosphorylates Bad or Caspase-9, promoting cell survival.

1.1.4. ROLE IN CANCER

The ability to proliferate in the absence of adhesion to the extracellular matrix (ECM) is a property of cancer cells and is termed “anchorage independence” (Schwartz et al., 1997). This property reflects the tendency of tumour cells to proliferate, survive and their ability to migrate beyond their original location into other tissues to form metastases (Brakenbusch et al., 2002). Therefore the relationship between tumour cells and the ECM strongly influences tumour development. An essential requirement for the survival of normal cells is cell attachment (Meredith et al, 1993). Complete loss of cell contact with substratum or adhesion to non-specific substratum such as poly-L-lysine induces apoptosis (Meredith et al., 1993; Frisch and Francis, 1994; Boudreau et al., 1996; Stupack et al., 2001; Bakre et al., 2002; Kim et al., 2002). As mentioned earlier, loss of contact does not however necessarily kill tumours, there are many examples of oncogenes or mutational changes in survival factors, such as upregulation of Bcl-2 or loss of p53 activity, which render the cells independent of survival signals (Jin and Varner, 2004).

A gene or gene locus in which mutation, loss of function or reduction in expression induces neoplasia is known as an oncogene. Oncogenes have the ability to overcome the mechanisms involved in normal cell processes. It is now well known that there is a connection between oncogenesis and integrins. As integrins are adhesion receptors which are involved in mediating the interactions between normal cells and tissues and the ECM they are essential for cell attachment and the control of cell migration, cell cycle progression and programmed cell death (Brakenbusch et al 2002). Therefore inappropriate changes in integrin expression and activation can have profound effects on tumorigenesis.

Invasion and metastasis

Anchorage independent tumour cells survive loss of contact with the substrate as a consequence of oncogenic activity such as mutations in survival factors. However, recent studies have shown that integrins can regulate survival. Stupack et al., (2001) and Kim et al., (2002) have shown that cell death can occur when a subset of integrins in a cell fail to bind their ECM ligands. Stupack et al., (2001), found that expression of $\alpha_5\beta_3$ or $\alpha_5\beta_1$ inhibited survival in cells attached to the matrix through other integrins. They also found that expression of dominant negative integrins such as Tac β_3 , inhibited survival by impairing normal integrin-mediated survival signalling. Suppression of Caspase 8 activity was found to be a consequence of integrin engagement whereas unligated integrins facilitated Caspase 8 activation and apoptosis (Kim et al., 2002). Therefore integrins can control cell survival; ligation of integrins in normal cells allows the cells to survive and apoptosis is initiated if the integrins do not engage (Jin and Varner, 2004).

The main cause of mortality from cancer is not due to primary tumours but to secondary tumours (Schwartz et al., 1997). A requirement for the formation of metastases is the process of cell migration. Initiation of migration occurs when the cell polarizes and the membrane protrusions form. These protrusions are fixed to the ECM via integrins and this process promotes interactions with the actin cytoskeleton triggering many different signalling molecules at the focal contacts. Cell contraction is then stimulated through integrin signalling allowing the movement of the cell on the adhesive contacts (Brakenbusch et al., 2002). This process is highly dependent on the integrins affinity or avidity for various ligands.

Increased expression levels of certain integrins have been recently reported to be associated with increased cell invasion and metastasis. $\alpha_5\beta_3$ has been found to be expressed on invasive melanoma but not benign or normal melanocytes (Gehlsen et al., 1992). Integrin α_6 has also been found to be significantly upregulated in numerous carcinomas, including head and neck and breast cancers (Garzino-Demo et al., 1998; Mercurio et al., 2001; Ramos et al., 20002).

Angiogenesis

The ongoing formation of new tumour vasculature is a key part of tumour progression. This process highlights another important way in which integrins modulate tumour growth and behaviour (Keely et al., 1998). Angiogenesis is the process by which new blood vessels develop from pre-existing vessels. Regulation of angiogenesis is carried out by growth factors and their receptors but also by the adhesive nature of the ECM. Several integrins therefore play important roles in regulating angiogenesis. One example of this is $\alpha_5\beta_3$, $\alpha_5\beta_3$ regulates the survival of newly sprouting blood vessels. Friedlander et al., (1995), showed that antibodies to $\alpha_5\beta_3$ caused the regression of some types of tumour in experimental animals by destroying the newly forming tumour vasculature. Both $\alpha_5\beta_3$ and $\alpha_5\beta_1$ are significantly upregulated on the endothelium during angiogenesis, and their expression partially controls angiogenesis (Brooks et al., 1999; Kim et al., 2000). The transcription factor Hox D3 controls these integrins' expression (Boudreau et al., 1997; Boudreau and Varner, 2003; Zhong et al., 2003), and is thought to be the switch to activate angiogenesis. Therefore integrins play important roles in regulating tumour angiogenesis.

Integrin inhibitors are thought to be possible therapeutic agents for the treatment of cancer. Antibody inhibitors for $\alpha_5\beta_3$, $\alpha_5\beta_5$ and $\alpha_5\beta_1$ are currently in clinical trials for the inhibition of tumour angiogenesis. Vitaxin (LM609) is a humanised monoclonal antibody against $\alpha_5\beta_3$ and reduces tumour-associated angiogenesis without affecting normal blood vessels. Vitaxin inhibits growth of human xenografts in mice and is being tested against a variety of cancers in patients (King, 2000).

1.1.5 INTEGRIN CROSS-TALK

Cross-talk is defined in the Merriam Webster Collegiate Dictionary as “unwanted signals in a communication channel (as in television, phone, radio or computer), caused by transfer of energy from another circuit (as by leakage or couplings)”. An “unanticipated event”, could also be added to this definition in terms of a biological signalling network. An example of authentic cross-talk is trans-activation of growth factor receptors by integrins. Trans-activation is the process where cell adhesion through integrins causes the phosphorylation of several growth factors, such as PDGF, FGF, VEGF, HGF and EGF. This process is therefore associated with the formation of complexes containing both integrins and growth factor receptors, (Schwartz and Ginsberg, 2002). c-Src, phosphoinositides and PKC are involved in cytokine and growth factor-dependent signalling. FAK, p130^{cas} and paxillin which localize to focal adhesions, mediate integrin signalling, connecting downstream to growth factor regulated pathways such as PI3-Kinase and MAP-kinase, (Schwartz et al., 1997). Moro et al., (1998), showed that an EGF receptor (EGFR) kinase inhibitor could block adhesion dependent activation of the ERK/MAP-kinase pathway in cells which expressed high levels of the EGFR. Pathways such as the PI3-Kinase or the MAPKinase pathway provide multiple points of intersection between integrins and growth factors, (Figure 1.1.5), (Zhou et al., 2003)

The purpose of integrin trans-activation has been postulated as a method of modulating rather than controlling growth factor signalling, (Schwartz and Ginsberg, 2002). However a possible downside to trans-activation could be the formation of tumours. Mistaking signals from integrins as signals from growth factors could lead to inappropriate cell growth leading to tumourigenesis, (Schwartz and Ginsberg, 2002). This process of crosstalk and transactivation between integrins and growth factors therefore needs to be examined in more detail and more understanding in this event, may lead to new discoveries into malignant transformation.

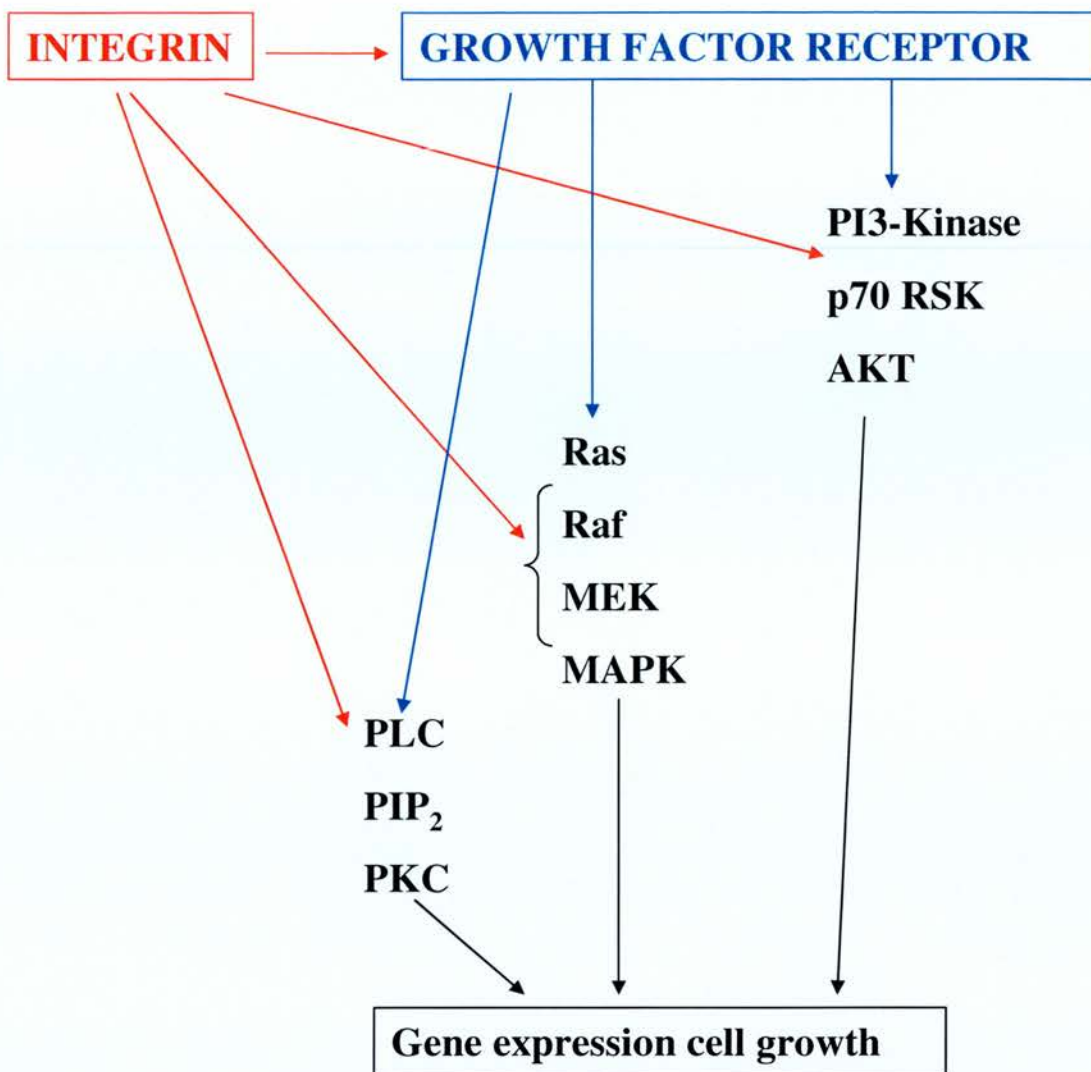


Figure 1.1.5 Crosstalk between integrin and growth factor receptors.

There are three steps in the process of convergence between integrin-mediated adhesion and signalling by growth factor receptor. These steps are: 1) autophosphorylation and activation of the receptors themselves; 2) hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce diacylglycerol and inositol triphosphate, leading to activation of protein kinase C (PKC); 3) activation of Raf and/or MEK in the MAP-kinase pathway and activation of PI3-Kinase, leading to activation of p70^{RSK} and AKT protein kinases. Figure adapted from Schwartz, 1997.

1.1.6 INTEGRIN-ASSOCIATED PROTEINS

Integrin Cytoplasmic domains

The cytoplasmic domain or tail of each subunit is actually the only portion of each integrin that is directly “seen” by the cytoplasm (Yamada, 1997). Inside-out signalling regulates the adhesive functions of integrins via the interaction of specific intracellular proteins with the integrin cytoplasmic tail. β cytoplasmic domains have been suggested to have an important role in targeting integrins to focal adhesions in a ligand –independent manner. From analyses of chimeric integrin receptors or mutant α and β subunits, β subunits have been shown to be necessary and sufficient for this function (LaFlamme et al., 1997). The α cytoplasmic domains regulate the specificity of the ligand-dependent interactions (Sastry et al., 1993; LaFlamme et al., 1994).

β cytoplasmic domains

As mentioned above β cytoplasmic domains are required for most aspects of integrin function, including cell adhesion and signal transduction events (Sastry and Horwitz, 1993). Chimeric receptors containing β cytoplasmic domains, connected to extracellular reporters such as IL-2 receptor extracellular domain (the Tac Ag) or the CD4 extracellular domain were sufficient to activate specific signalling events (Akiyama et al., 1994; Lukashev et al., 1994). β cytoplasmic domains have also been shown to function as dominant negative mutants, and inhibit endogenous integrin functions such as fibronectin matrix assembly, cell spreading and cell migration (LaFlamme et al., 1994). Several cytoskeletal and signalling proteins have been found to bind to integrin β cytoplasmic domains as shown in table 2.

<u>Cytoplasmic proteins</u>	<u>Integrin cytoplasmic interaction</u>	<u>Function</u>
Cytohesin-1	$\beta 2$	A guanine nucleotide exchange protein. Overexpression increases $\alpha_L\beta_2$ adhesion.
pp59^{ILK}	$\beta 1$, $\beta 2$ and $\beta 3$	Overexpression inhibits cell adhesion to fibronectin, laminin, vitronectin.
$\beta 3$-Endonexin	$\beta 3$	Overexpression activates $\alpha_{IIb}\beta_3$.
Calreticulin	α subunits	Interacts with $\alpha_2\beta_1$ and overexpression increases cell spreading and decreases motility.
CIB	α_{IIb}	Calcium binding protein.
ICAP-1	$\beta 1$	Ligation of $\beta 1$ integrins induce ICAP-1 phosphorylation.
pp125^{FAK}	β subunits	A tyrosine kinase which localizes to focal adhesions and overexpression induces increased migration.
Filamin	$\beta 1A, \beta 2, \beta 3$	Structural cytoskeletal protein
Talin	β, α_{IIb}	Structural cytoskeletal protein
Paxillin	β subunits	Structural cytoskeletal protein

Table 2. Cytoplasmic proteins which interact directly with integrin cytoplasmic domains.

Table adapted from Fernandez et al., 1998; Hughes and Pfaff, 1998; Brown, 2002.

Interactions of proteins with integrin cytoplasmic domains.

Cytohesin-1, is a cytoplasmic protein which has been shown to be a guanine nucleotide exchange protein for ADP-ribosylation factor (ARF) responsible for the formation of active ARF-GTP (Meacci et al., 1997). However it has also been shown to regulate β_2 integrins (Table 2); when overexpressed, $\alpha_L\beta_2$ -dependent binding to ICAM-1 was induced (Kolanus et al., 1996). Cytohesin contains a PH domain which binds to 3-phosphorylated phosphoinositides and a sec 7 domain which binds to the β_2 tail (Shattil et al., 1990).

The serine threonine kinase p59^{ILK} has also been shown to bind to integrin β_1 , β_2 and β_3 cytoplasmic tails. When overexpressed, p59^{ILK} disrupts cell-substratum adhesion (Hannigan et al., 1996).

β_3 -endonexin, which is specific for β_3 tails (Shattil et al., 1995), has been shown to regulate the affinity state of $\alpha_{IIb}\beta_3$ when co-transfected into CHO cells and analysed by FAC's (Kashiwagi et al., 1997).

Focal adhesion kinase (pp125^{FAK}) and paxillin have been shown to interact directly with integrin cytoplasmic domains (Shaller et al., 1995). The N-terminal domain of FAK has been shown to bind directly to the cytoplasmic tail of the β_1 subunit. Paxillin and other cytoplasmic proteins such as F-actin, talin, tensin and vinculin have all been shown to associate directly or indirectly with integrin cytoplasmic domains, connecting the actin cytoskeleton with the integrin cytoplasmic domain (Fernandez et al., 1998).

The cytoskeletal protein talin, binds to most integrin β -tails (Calderwood et al., 2000). It has been shown to have a modulating effect on integrin activation. Talin binding to integrin β tails activates integrins (Calderwood et al., 1999, 2002; Kim et al., 2003), and if this interaction is disrupted integrin activation is prevented (Tadokoro et al., 2003). Calderwood et al., (2004) have now shown that competition for talin results in trans-dominant inhibition of integrin activation. This trans-dominant inhibition of integrin activation is one form of integrin cross-talk and occurs when ligand binding to

one integrin inhibits the activation of a second integrin. This process may have a role in normal physiology, such as leucocyte transmigration, platelet adhesion and control of angiogenesis. This finding could have implications for the mechanisms of drugs that target integrins (Reiderer et al., 2002; Schwartz and Ginsberg, 2002; Hynes, 2002).

A number of calcium binding proteins or calcium dependent proteins have been reported to associate with integrin cytoplasmic domains. An example of these proteins is Calpain, which is a calcium dependent protease, and thought to cleave the cytoplasmic domains of integrins. It has now been reported that it regulates migration. Huttenlocher et al., (1997) demonstrated that $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ mediated migration in CHO cells could be blocked when Calpain inhibitors were used in the system. Another protein thought to interact with integrin cytoplasmic domains is Calreticulin. Calreticulin is an intracellular calcium binding protein and has been shown to interact with a highly conserved GFFKR motif present in the α subunit cytoplasmic domains. It has been suggested to associate with active but not inactive $\alpha_2\beta_1$ and acts by maintaining the integrin in a high affinity state, primed for ligand binding (Coppolino et al., 1995).

ICAP-1(Integrin cytoplasmic domain associated protein-1) is another protein thought to have a role in the regulation of affinity or avidity modulation. Chang et al., (1997), reported that ICAP-1 binds specifically to the β_1 cytoplasmic domain via a NPXY motif.

<u>Membrane anchored proteins</u>	<u>Integrin cytoplasmic interaction</u>	<u>Function</u>
IAP	$\beta 3$	Thrombospondin-1 receptor.
uPAR	$\beta 1, \beta 2$ and $\beta 3$	Association required for cell migration, tumour invasion and host defense.
TM4SF (CD9, CD81, CD151 etc.)	$\beta 1, \alpha 4\beta 7, \alpha L\beta 2$	Precise function unknown at present but may play a role in adhesion, motility and aggregation via integrins
MMP-1	$\alpha 2\beta 1$	ECM degradation, possible members of a supramolecular complex.
MMP-2	$\alpha 5\beta 3$	ECM degradation, possible members of a supramolecular complex.
CD98	$\beta 1$	Amino acid transporter, regulator of integrin function.

Table 3 . Membrane anchored and GPI-linked proteins which interact directly with integrin cytoplasmic domains.

Table adapted from Fernandez et al., 1998; Porter and Hogg, 1998; Brown, 2002.

Membrane-Anchored proteins

Many transmembrane or GPI-linked membrane proteins have also been shown to bind to integrins, via immunoprecipitation experiments or colocalization analysis using fluorescence microscopy, (Table 3). It has been clear over the past several years that integrins do not only work as individual receptors but also as components of supramolecular complexes at the plasma membrane (Brown, 2002).

Possible components of these supramolecular complexes are matrix metalloproteinases (MMP's). MMP's are secreted proteins but have been found to bind to cell-surface integrins. MMP-2 has been shown to interact with $\alpha_5\beta_3$ (Brooks et al., 1998), and when this association is blocked, angiogenesis is inhibited. Silletti et al., (2001) reported that this inhibition indicated a physical association between integrin and protease is required for angiogenesis and enhancement of endothelial proliferation or migration.

GPI-linked proteins (glycan phosphatidylinositol-linked) also associate with integrins. An example of this association is with Urokinase-type plasminogen activator receptor (uPAR). uPAR is a heavily glycosylated cell surface receptor which binds to urokinase and vitronectin (Chapman, 1997). uPAR and β_1 integrins interact directly as a consequence of a conformational change in the integrin and lead to an inhibition of adhesion to fibronectin (Wei et al., 1996). uPAR has also been shown to interact with β_2 integrins activated by mAbs (Fernandez et al., 1998).

CD47, also known as IAP (integrin associated protein), is a receptor for thrombospondin. It spans the membrane five times and co-immunoprecipitates with β_3 integrins. It has been shown that CD47 binding to thrombospondin induces the activation of the platelet integrin $\alpha_{IIb}\beta_3$ (Lindberg et al., 1994; Chung et al., 1997). CD47 has also been shown to associate with $\alpha_5\beta_3$. This association is dependent on cholesterol. It is thought that this cholesterol is required for a conformational change to the molecule CD47 in order for CD47 to associate with $\alpha_5\beta_3$ (Green et al., 1999).

Tetraspanin molecules such as CD9, (a member of the transmembrane 4 superfamily which spans the membrane four times) have also been shown to have an integrin association. CD9 has been shown to co-localize with $\alpha_{IIb}\beta_3$ in platelet α -granule membranes and filopodia (Brisson et al., 1997). Gutierrez- Lopez et al., (2003) reported the presence of a functionally relevant epitope on CD9 whose expression depends on CD9's association with $\alpha_6\beta_1$.

CD98 is a 125kDa heterodimeric transmembrane glycoprotein amino acid transporter. It has now been recently implicated as a regulator of integrin activation, using a screening strategy based on identifying proteins that overcome dominant suppression (Fenczik et al., 1997). It has also been shown to associate with integrins through the β_{1A} cytoplasmic tail (Zent et al., 2000). This association modulates integrin avidity and adhesion (Fenczik et al., 1997; Warren et al., 2000). Fenczik et al., (1997), also found that crosslinking CD98 using monoclonal antibodies stimulated β_1 -dependent adhesion. These studies indicated that CD98 is involved in the regulation of ligand binding activity of integrins. Therefore for my thesis I wanted to explore the exciting link between CD98 and β_1 integrins further and this subject is discussed in more detail in section 1.2.

1.2 CD98

CD98 is a heterodimeric glycoprotein which is ubiquitously expressed on cell surfaces and is highly conserved between species. It was discovered in 1981 by Haynes et al., while studying monoclonal antibodies reactive with lymphocyte cell surface differentiation antigens. It was previously known as 4F2 but was later given a cluster designation (CD) number at the Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens (Diaz et al., 1995). CD98 has been shown to have many functional effects in processes such as adhesion, fusion and amino acid transport. It is also highly upregulated following cellular activation. However no definitive role for CD98 in cell activation has been established.

1.2.1 MOLECULAR STRUCTURE

CD98 is a disulphide linked 125 kDa heterodimeric type II transmembrane glycoprotein. It is composed of a glycosylated heavy chain of 80 kDa and a non-glycosylated light chain of ~40 kDa (Haynes et al., 1981).

Heavy Chain

Three separate laboratories, Lumadue et al., 1987; Quackenbush et al., 1987 and Texeira et al., 1987, have cloned the cDNA for the heavy chain of CD98. The sequence was predicted to encode a glycosylated protein of 529 amino acids. Amino acids 83-106 encode a single transmembrane segment. The CD98 protein was found to have an extracellular carboxyl terminus and an internal amino terminus. This orientation is shared by other type II transmembrane proteins, eg. the transferrin receptor, the HLA-associated invariant chain (Bertran et al., 1992) and CD69.

The primary sequence contains four potentially glycosylated asparagine residues which are located in the carboxy-terminal half of the protein (Deves and Boyd, 2000).

Cys109 has been shown to participate in disulphide bond formation with the light chain (Pfeiffer et al., 1998; Torrents et al., 1998) as shown in figure 1.2.1.

The murine (Parmacek et al., 1989), rat (Broer et al., 1997) and hamster (Fenczik et al., 1997) CD98 heavy chain have been cloned and showed a high level of sequence identity to human CD98 heavy chain (CD98HC).

Light Chains

The CD98 light chains consists of 502-535 amino acids and are predicted to be very hydrophobic proteins with 11 or 12 putative transmembrane domains (Nakamura et al., 1999). These light chains are linked to the CD98 heavy chain through the extracellular cysteine, Cys109, which participates in disulphide bond formation (Pfeiffer et al., 1998). So far 7 cDNA's encoding distinct chains have been identified.

LAT-1, y^+ LAT-1, y^+ LAT-2, xCT and LAT-2 have all been shown to be linked with the CD98HC (Okamoto et al., 2002). The heterodimer composed of LAT-1 and CD98HC is known to be responsible for sodium independent neutral amino acid transport (L-type transport). The LAT-1-CD98HC heterodimer is found ubiquitously and is highly expressed in proliferating tissues, in particular tumour tissues, suggesting a role in amino acid transport (Meier et al., 2002). LAT-2-CD98HC is found only in tissues containing epithelial barriers (Bassi et al., 1999; Pineda et al., 1999; Rossier et al., 1999; Segawa et al., 1999; Rajan et al., 2000). LAT-2 transports large and small neutral amino acids with a lower affinity than LAT-1 (Pineda et al., 1999; Segawa et al., 1999). y^+ LAT-1 and y^+ LAT-2 are associated with y^+ L- type transport which transfers basic amino acids in a sodium-independent manner and neutral amino acids in a sodium dependent manner. The xCT-CD98HC heterodimer transports cysteine/glutamate (Okamoto et al., 2002).

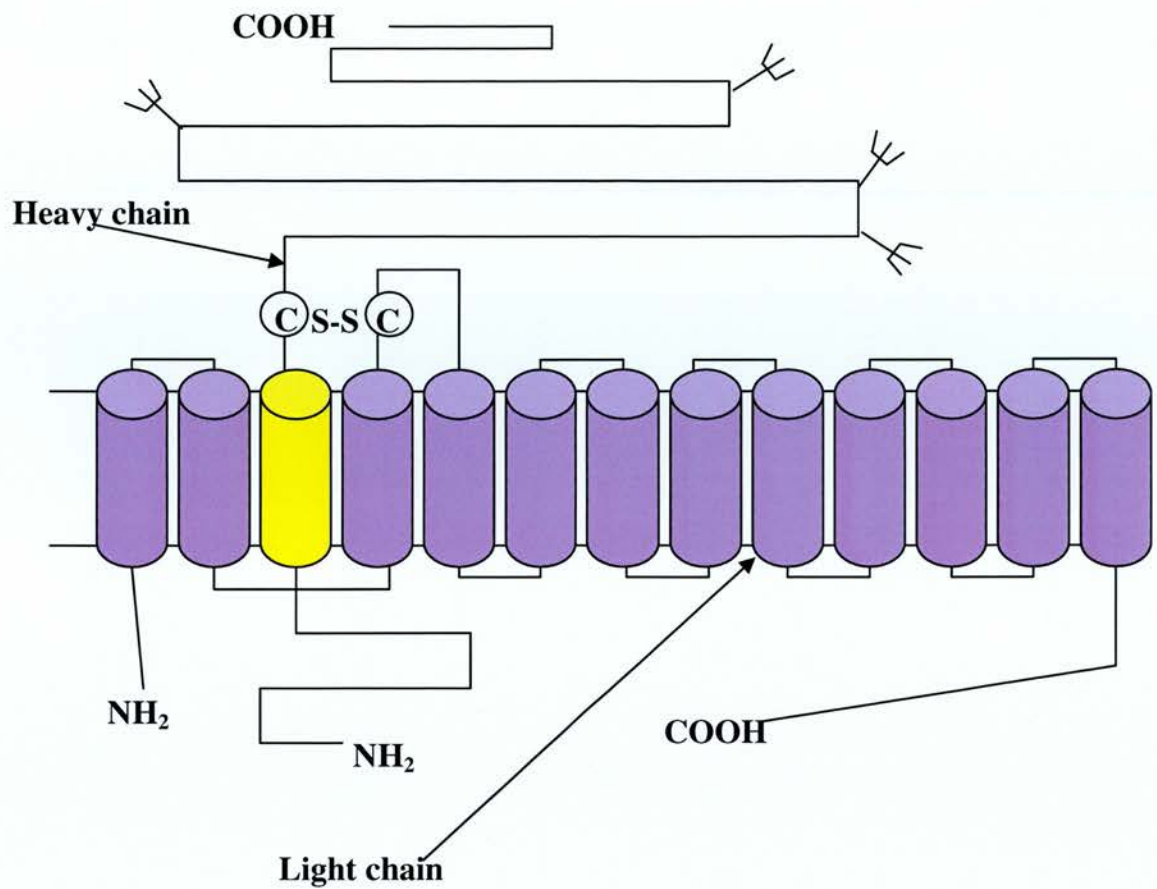


Figure 1.2.1 Proposed model of CD98.

The heterodimer is composed of a glycosylated heavy chain (yellow) and a light chain with 12 transmembrane domains (purple). The two types of subunits are connected by a disulphide bond. Redrawn from Mastroberardino et al., 1998; Deves and Boyd, 2000.

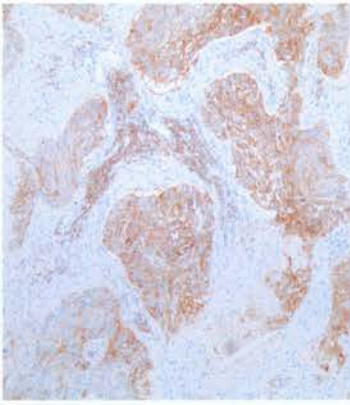
1.2.2 THE GENE

The CD98HC is located on the long arm of chromosome 11 and is composed of nine exons and spans approximately 8 kb (Diaz et al., 1998). The expression of CD98HC is partially regulated by a 5' upstream G+C rich binding sites with four potential Sp1 (transcription factor) binding sites (Deves and Boyd, 2000). No studies have been carried out on the genes encoding the light subunits. However Nakamura et al., (1999), indicated that the CD98HC is expressed on the cell surface as a monomer on its own, whereas the CD98LC is transported to the plasma membrane only in the presence of the CD98HC.

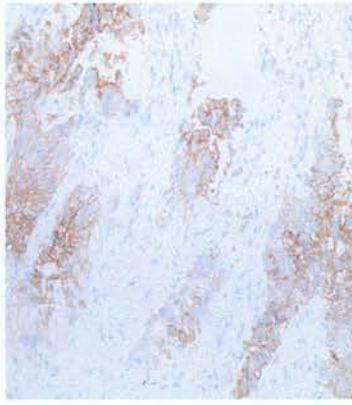
1.2.3 TISSUE EXPRESSION

CD98 is ubiquitously expressed and highly conserved between species. It is expressed at low levels on the surface of quiescent cells but is rapidly upregulated following cellular activation. This level of expression remains elevated until the cell cycle is complete (Azzarone et al., 1985; Suomalainen et al., 1986; Parmacek et al., 1989). CD98 is strongly expressed on human embryonic fibroblasts but this expression gradually diminishes from 100 to 1% on fibroblasts from normal adults (Azzarone et al., 1985). CD98 has been found to be highly expressed on many tumour cell types (Bellone et al., 1989; Dixon et al., 1990) as shown in figure 1.2.3. Increased CD98 expression has also been shown to have a correlation with the development, progression and metastatic potential of tumours (Esteban et al., 1990; Garber et al., 2001; Yoon et al., 2003).

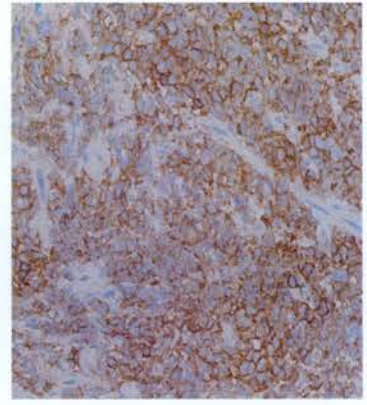
LAT-1 message is highly expressed in the brain, spleen, and placenta and at very low levels in epithelial cells and adult liver (Kanai et al., 1998; Prasada et al., 1999; Nakamura et al., 1999). LAT-2 is highly expressed in the kidney and placenta and at lower levels in liver, skeletal muscle and heart (Pineda et al., 1999). Human y+ LAT-1 can be detected in kidney and at high levels in peripheral blood leukocytes (Pfeiffer et al., 1999).



Squamous Cell Carcinoma



Adenocarcinoma



Small Cell Lung Cancer

Figure 1.2.3. CD98 is highly expressed in tumour tissues.

Photographs showing various tumour tissues. Expression of CD98 is shown here by areas of brown staining. CD98 is highly expressed in Small Cell Lung Cancer.

Pictures courtesy of N Henderson.

1.2.4 FUNCTIONS

Fusion

Membrane fusion is an important process in many cellular functions such as exocytosis, endocytosis, organelle formation and intracellular trafficking. Multinucleated giant cells are considered to originate from the fusion of monocyte-macrophage lineage cells and have been induced *in vitro* from human blood monocytes (Anderson, 2000). Multinucleated giant cells can be classified into two groups, Langhans' giant cells or foreign body giant cells (FBGC's). Langhans' giant cells are commonly seen in infective granulomatous diseases such as tuberculosis and schistosomiasis and the granulomatous disorder sarcoidosis. FBGC's are most commonly found in foreign body granulomas. However in addition to these two groups, the osteoclast and HIV-1 induced CD4⁺ T cell derived syncytia are also considered multinucleated giant cells.

Ito et al., 1987 previously found that syncytium formation in Newcastle Disease Virus (NDV)-infected L929 cells was enhanced after addition of anti-L929 anti-serum to culture medium. This finding suggested that there were factors present on the cell surface of host cells capable of regulating virus-mediated cell fusion. Monoclonal antibodies capable of enhancing cell fusion were isolated and 2 molecules gp80 and gp135 were immunoprecipitated. These molecules were designated fusion regulatory proteins, FRP-1 and FRP-2 respectively. They were found to induce HIV-mediated cell fusion. When U2ME-7 cells, a cell line transfected with the HIV gp160 gene, were cultured in the presence of anti-FRP-1 mAb or anti-FRP-2 mAb, formation of syncytium occurred (Ohta et al., 1994). Tabata et al., (1994), also found that antibodies raised against FRP-1 could also enhance cell fusion induced by the measles virus. Ohgimoto et al., (1995) then reported that eleven of fifteen N-terminal amino acids of FRP-1 were the same as the amino acid sequence of the CD98 heavy chain. The functional properties of FRP-1 were also very similar to those of CD98. They found that HBJ127 (heavy chain sub-unit of CD98 mAb) induced multinucleated giant cells in U2ME-7 cells and

treatment of monocytes with other antibodies (4F2, H227) directed against CD98 resulted in the formation of polkaryocytes. Ohgimoto et al., (1996) also found that by modifying the FRP-1 system, the HIV-mediated cell fusion could be regulated. This was demonstrated by FRP-1 (CD98HC) monoclonal antibodies either suppressing or inducing multinucleated giant cell formation, indicating that FRP-1/CD98HC is a multipotential molecule. This finding was further clarified by work carried out by Okamoto et al., (1997). They found that anti-FRP-1 antibodies which enhanced cell fusion in Newcastle Disease Virus infected Hela cells, delayed human parainfluenza virus type 2 (HPIV-2) induced cell fusion. Both of these viruses belonged to the same genus, *Rubulavirus* indicating that anti-FRP-1 antibodies could not only enhance/induce but also inhibit/delay virus induced fusion, suggesting that CD98 is a multi-functional molecule. This was further confirmed when the same authors using mutated CD98 constructs, caused dominant negative suppression of CD98HC induced fusion. The CD98 constructs either had the cytoplasmic domain replaced with the cytoplasmic domain of human parainfluenza virus type 2 (HPIV2) haemagglutinin-neuramidase (HN) or the FRP-1/330 cysteine was mutated to serine. Anti-FRP-1 antibodies enhanced NDV polkaryocyte formation in Hela cells whereas in NDV- infected cells constitutively expressing FRP-1/HN, anti-FRP-1 antibodies had no effect on polykaryocyte formation. These constructs proved that an intact CD98 molecule is required for viral-mediated cell fusion. The authors also speculated that the dominant negative suppression was a consequence of sequestering the CD98 light chain. The mutation (C330A) abolishes the covalent bond link between the heavy and light chain and also inhibited viral induction of fusion.

FRP-1/CD98HC is expressed selectively on monocytes but is absent on other PBMC's and granulocytes (Tabata et al., 1994). Anti-CD98HC monoclonal antibodies have been shown to promote $\beta 2$ integrin activation stimulating monocyte cell-cell aggregation. This subsequently activates $\beta 1$ promoting cell fusion and polykaryocyte formation. This effect however could be blocked by a $\beta 2$ antibody (Ohta et al., 1994) suggesting that integrin activation may be involved in the effect of the antibody. This

was clarified by work carried out by Fenczik et al., (1997), on the role of CD98HC on integrin activation.

Kudo et al., (2003) showed by manipulating CD98 expression by transfection with the anti-sense oligonucleotide, that the rate of cell fusion is suppressed in placental syncytiotrophoblast formation. They also showed that following fusion, amino acid influx activity through system L is inhibited by anti-sense oligonucleotides to CD98. This indicates that there is a possibility that amino acid transport plays a role in cell fusion and that the CD98LC may be involved in the induction of cell fusion through amino acid transport.

FRP-1/CD98HC antibodies have also been found to be capable of fusing blood monocytes giving rise to multinucleated cells which express structural and functional properties of osteoclasts (Higuchi et al., 1998). The osteoclast is responsible for bone resorption and membrane fusion is the first step of osteoclast formation. Higuchi et al., (1998,1999), found that when human blood monocytes were incubated in the presence of anti –FRP-1/CD98HC mAb, TRAP (tartrate resistant acid phosphatase, an osteoclast marker)-positive multinucleated giant cells appeared present. Mori et al., 2001 found that an inhibiting CD98HC mAb (HBJ127) blocked cell fusion but also suppressed RANKL (receptor activator of NF- κ B ligand)-induced cell fusion and this indicated that there is cross-talk between the CD98 mediated and RANKL-mediated osteoclastogenesis. However how NF- κ B pathways are involved in CD98 function is unknown at present.

Integrins and Intracellular signalling

In addition to a possible involvement in NF κ B and RANKL-induced osteoclastogenesis, CD98 has also been shown to have a role in other intracellular signalling pathways.

Warren et al., (1996), obtained evidence that CD98 could transduce signals that manifested in both the tyrosine phosphorylation of a ~125kD protein and the induction of homotypic aggregation in a Pro-T cell progenitor line. They elucidated this using a

Joro177 monoclonal antibody which they found in their search for cell surface markers expressed on hematopoietic stem cells. By searching in databases such as BLASTn, BLASTx and GAP programs they showed that Joro 177 cDNA had 77.5% nucleotide sequence identity, 76.4% and 86.4% amino acid sequence identity and similarity to the heavy chain of CD98.

The involvement in intracellular signalling was further confirmed by work carried out by Fenczik et al., (1997), on the role of CD98 in integrin activation. By designing a new genetic cloning strategy based on changes in integrin affinity, Fenczik et al., (1997), identified a potential modulator of integrin activation. They used a CHO cell line stably expressing a chimaeric integrin ($\alpha_{2b} \alpha_{6A} \beta 3 \beta 1$) that contains the extracellular and transmembrane domains of $\alpha_{2b} \beta 3$ fused to the cytoplasmic domains of $\alpha_{6A} \beta 1$. This chimaeric integrin was activated through the $\alpha_{6A} \beta 1$ but had the ligand-binding properties of $\alpha_{2b} \beta 3$. In CHO cells this chimaeric integrin is constitutively active. When isolated integrin $\beta 1$ cytoplasmic domains (Tac $\beta 1$ chimera) were overexpressed, integrin signalling was suppressed however when $\alpha \beta$ py cells were co-transfected with a CHO-cell cDNA expression library and Tac $\beta 1$, FACs analysis produced cDNA's which complemented (rescued) dominant suppression. 5F8 was a single cDNA, and was found to be able to reverse Tac $\beta 1$ suppression. After a BLAST database search, 5F8 was found to be 72% identical to the heavy chain of 4F2/CD98. Having identified CD98 as a potential modulator of integrin activation they investigated CD98's role in integrin function. They found that the anti-CD98 monoclonal antibody 4F2 markedly enhanced SCLC cell adhesion to both laminin and fibronectin. Fab fragments of 4F2 did not enhance SCLC adhesion suggesting therefore that clustering of CD98 is required for 4F2-induced upregulation of $\beta 1$ function.

In addition, Chandrasekaran et al., (1999), showed that the $\alpha 3 \beta 1$ integrin mediated adhesion of MDA-MB435 and MDA-MB231 breast cancer cells to thrombospondin-1. Thrombospondin (TSP-1) is an extracellular matrix glycoprotein which has diverse effects on cell behaviour. Via the integrin $\alpha 3 \beta 1$ they also showed that

adhesion to laminin and TSP-1 could be stimulated by exogenous stimuli such as the ligation of CD98 and IGF-1.

The role CD98 plays in integrin activation was investigated further by Zent et al., (2000). They reported that CD98 associated with integrin β cytoplasmic domains and that this association occurred in a specific manner. CD98 only interacted with integrin β cytoplasmic domains which were a particular class and splice variant. In particular CD98 associated with $\beta 1A$ but not $\beta 1D$ or $\beta 7$ integrin cytoplasmic domains. This group also showed that the capacity of CD98 to overcome dominant suppression of integrin activation correlated with its capacity to bind to integrin tails. Many groups have been unable to crosslink the CD98HC to $\beta 1$ integrins however Merlin et al., (2000), showed that CD98 could selectively co-immunoprecipitate with both LAT2 and $\beta 1$ integrins and were all polarized to the basolateral domain. They concluded that by associating with $\beta 1$ integrins, CD98 may influence $\beta 1$ integrin distribution and function. Cho et al., (2001), confirmed that there was an important interaction between CD98 and $\beta 1$. They revealed this by analysing their respective aggregation properties. The aggregation produced CD98 and $\beta 1$ shared many properties, suggesting that the heterogeneity between the aggregation induced by CD98 and $\beta 1$ reflected the presence of specific conformational epitopes on the CD98 molecule and indicated that only some of these are involved in inducing aggregation. However there were also differences between the induced aggregational properties of CD98 and $\beta 1$, CD98 induced aggregation was EDTA insensitive compared to that of $\beta 1$ induced aggregation therefore, suggesting that CD98 did not require divalent cations and implied that $\beta 1$ plays a role in the inductive phase of the response rather than mediating the actual cell-cell adhesion.

Warren et al., (2000), also found evidence of a functional association between CD98 and integrins in lymphocytes. They found that uniquely for a non-integrin, CD98 co-stimulation could be blocked by an antibody to $\beta 1$ integrin. They previously showed that T-cell co-stimulation mediated by integrins $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 4\beta 7$ and $\alpha L\beta 2$ could be partially prevented by a $\beta 1$ integrin specific monoclonal antibody 18D3. They found that co-stimulation mediated by non-integrins was unaffected; however in 2000 they reported

that CD98 was uniquely sensitive and affected by the inhibitory effects of $\beta 1$ blocking antibodies.

Kolesnikova et al., (2001), demonstrated that CD98 constitutively and specifically associated with intact $\beta 1$ heterodimers. They showed that the CD98-integrin interaction occurred in the context of low density protein-lipid microdomains, suggesting that lipid rafts may be involved. Kolesnikova also demonstrated that the CD98 light chain might be necessary for CD98-integrin association by using mutants of extracellular cysteines. These cysteines were mutated to serine, C330S or C109S. C330S retained association with $\beta 1$ in NIH 3T3 cells but C109S (cysteine required for disulphide linkage to the light chain) showed no detectable $\beta 1$ association. However this finding was in disagreement with that of Fenczik et al., (2001). By using chimeras of CD98HC and a type II transmembrane glycoprotein CD69, they examined the relationship between CD98HC's ability to stimulate amino acid transport and affect integrin function. They reported that mutations of cysteines which disrupt CD98HC and light chain association had no effect on integrin activation and did not disrupt its binding to $\beta 1A$. They also showed that the cytoplasmic domains and transmembrane domains of CD98HC were necessary and sufficient for effects on integrin function. However if those domains were replaced with those of CD69, while affecting integrin function, these replacements had no effect on the amino acid transport function of CD98HC. This therefore indicated that the amino acid transport activity of CD98HC is not required for its effects on integrin function.

By clustering $\beta 1$ integrins, Rintoul et al., (2002), proposed that CD98 acts like a "molecular facilitator", when crosslinked resulting in integrin activation and adhesion. They showed that CD98 and $\beta 1$ integrin were constitutively colocalized in SCLC cells regardless of integrin activation state and cell polarization. They also found that by crosslinking CD98, PI3-kinase, its product PIP3 and its down stream effector PKB/AKT were stimulated. This finding was in agreement with reports by Ito et al., (1992) and Warren et al., (1996), in which they showed that tyrosine kinase activation mediated

CD98 homotypic aggregation. PI3-kinase can be activated by receptors with intrinsic tyrosine kinase activity.

There is increasing evidence that CD98 can regulate different types of adhesion molecules through distinct mechanisms. Kakugawa et al., (2003), demonstrated that anti-CD98 antibodies could induce rapid cell aggregation in BaF3 cell lines expressing CEA-CAM1, but not in parental BaF3 cells. A significant portion of CEA-CAM could also be co-immunoprecipitated by anti-CD98 mAb and CEA-CAM1 and CD98 were also found to be colocalized at the cell adhesion sites. These results suggested that CD98 stimulation could activate the cell adhesion mediated by CEA-CAM1. This finding supported the idea that CD98 acts as a “molecular facilitator” in the plasma membrane.

A molecule which plays a role in integrin-dependent cell adhesion is Rap-1. Suga et al., (2001), showed that the Ras-related GTPase known as Rap-1 can be activated by crosslinking CD98 which stimulates LFA-1 through the PI3-kinase pathway. The activation of LFA-1 via CD98 was completely inhibited by PI3-kinase inhibitors such as wortmannin and Ly294002. This suggested the involvement of PI3-kinase pathway and the involvement of “inside-out” signalling for integrin activation. They proposed that LFA-1 activation by PI3-Kinase generates active Rap-1 GTP, both mediating cytoskeletal cell adhesion and increasing $\beta 2$ activation in forming a positive feedback loop.

The physical association between CD98 and $\beta 1$ has recently been further confirmed by work carried out by Miyamoto et al., (2003) on T-lymphocytes. They showed that $\beta 1$ integrin and CD98 colocalized to the same region of a T-lymphocyte and demonstrated that $\beta 1$ could be reprecipitated from CD98 complexes from T-lymphocytes lysates.

All of these findings implicate a role for CD98 in integrin function and suggest that CD98 may be a member of macro-molecular signalling complex. Integrin-mediated interactions are required for many essential processes such as inflammation, cell adhesion, migration, proliferation and differentiation. Therefore abnormal regulation of integrin function may result in a disruption in growth control and altered cell function.

This is confirmed by work carried out by many groups in which they have investigated the role in which CD98 plays in cancer and tumorigenesis.

Transformation

Anchorage independence is a phenomenon which enables malignant tumour cells to survive and grow. Oncogenes are genes or gene locus in which mutation, loss of function or aberration in expression induces transformation. There is now increasing evidence that CD98 has oncogenic potential. Parmacek et al., (1989); Nakamura et al., (1999), reported that the heavy chain of CD98 is expressed in most tissues and is present in all cell lines and tumour cells. In addition, increased CD98 expression correlates with the development, progression and metastatic potential of tumours (Esteban et al., 1990; Garber et al., 2001; Yoon et al., 2003). Recently Yoon et al., (2003), examined the alterations in CD98HC expression levels in hamster buccal cancers. They found that the CD98HC expression level increased in correlation with the hamster buccal pouch malignant progression.

In 1999, Hara et al., demonstrated that overexpression of CD98 induced anchorage independent growth of NIH 3T3 cells. This was examined by testing the ability of human CD98-transfected clones to grow in soft agar, as this is a measurement of malignancy. This group also showed that overexpression of CD98 also caused tumours to form in athymic mice and that the degree of malignancy of CD98-transfected clones was proportional to the expression levels of CD98. In addition, in 2000, Hara attempted to characterize the functional domains of CD98HC in terms of cellular proliferation and malignancy. This was carried out by analysing the effects of truncation of the extracellular domain. They found that expression of CD98HC with truncated extracellular domain was sufficient to transform mammalian fibroblasts. These results suggested that the extracellular domain of CD98HC played a regulatory role in the process of malignant transformation.

Shishido et al., (2000) examined whether the light chain of CD98 was required for transformation capabilities of the CD98HC. They showed in BALB 3T3 cells, that

the ability to transform by CD98 was lost when C103 was replaced with a serine residue, resulting in inability to associate with the light chain. These results suggested that the association between exogenous CD98HC and endogenous CD98LC at the cell surface was necessary for anchorage-independent growth induced by the transfection of CD98HC. They also found that Cysteine 103 of the CD98HC is involved in the disulphide linkage, which is in accordance with studies by Nakamura et al., (1999); Pfeiffer et al., (1998). However the debate whether the CD98LC is required for CD98 functions other than amino acid transport goes on.

Recently Rintoul et al., (2002), demonstrated that crosslinking CD98 can promote anchorage independent growth in SCLC cells. These findings are producing more and more evidence that CD98HC has oncogenic potential and plays a role in tumourgenesis.

1.2.5 GALECTIN 3

The possible ligands for CD98 remain to be determined. One possibility is Galectin 3. Galectin 3 is a lectin that recognises β -galactosidase containing carbohydrates. It is secreted from the cytoplasm of macrophages, eosinophils, neutrophils and mast cells and then binds with appropriately glycosylated proteins either at the cell surface or in the extracellular matrix. It then plays a role in cell signalling, triggers superoxide production in neutrophils and potentiates IL-1 production by monocytes (reviewed by Deves and Boyd, 2000).

Sato et al., (1992), showed that galectin 3 bound to matrix glycoproteins such as tumour derived laminin and foetally derived fibronectin and blocked attachment and spreading of cells to these matrices, suggesting a possible role for steric hindrance of cell binding and modulation of integrin binding. Dong and Hughes, (1996) showed exogenous galectin 3 bound to the surface glycoproteins, including CD98 of human Jurkat T cell line.

However to date, no direct link between galectin 3 and CD98 and biological function has been discovered.

1.2.6 CALCIUM

Some evidence suggests that CD98 is involved in the regulation of intracellular Ca^{2+} concentration via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. There is also evidence linking calcium and galectin 3, producing another possible link between CD98 and galectin 3. Posillico et al., (1987), showed that incubating human parathyroid adenoma cells with the monoclonal antibody 4F2 triggered a transient increase in intracellular Ca^{2+} and a decrease in parathyroid hormone secretion. Dong and Hughes, (1996), as well as demonstrating a possible link between CD98 and galectin 3 also showed that galectin 3 binding to cell surface glycoproteins lead to an increase in intracellular calcium. However R.Rintoul (unpublished data) showed that stimulation with the CD98 monoclonal antibody 4F2 did not cause an increase in SCLC intracellular Ca^{2+} concentration.

1.2.7 TROPOMYOSIN AND OTHER CYTOSKELETAL PROTEINS

Shishido et al., (2000), reported that there may be a physical association between CD98 and tropomyosin isoforms either directly or indirectly and that there may be a possible functional relevance in cell-cell adhesion. This work confirmed findings by the same group in which they found that vimentin, tropomyosin and heat shock cognate protein 70 (hsc70), associated with FRP-1. By using monoclonal antibodies prepared against immunoaffinity –purified FRP-1 complex and subsequently gene cloning using a Hela lambda gt11 cDNA library (Suga et al., 1995).

1.2.8 NCAM (NEURAL CELL ADHESION MOLECULE)

The neural cell adhesion molecule (NCAM) is a member of the Ig superfamily. It plays a pivotal role in neural development and synaptic plasticity (Ronn et al., 1998 and Berezin et al., 2000). Three isoforms of NCAM are generated through alternative

splicing, NCAM-120, NCAM-140 and NCAM-180. NCAM engages in homophilic (NCAM-NCAM) and this has been suggested to induce neurite outgrowth (Kolkova et al., 2000) . New evidence has now come to light, showing that NCAM can also exert heterophilic interactions with various molecules such as the cell adhesion molecule L1, members of the FGFR family and signal transducing molecules such as FAK and p59^{fyn} (Cavallaro et al., 2001 and Seidenfaden et al., 2003). NCAM also plays an important role in tumour progression. In many cancers, the expression of NCAM changes from NCAM-120 to the transmembrane isoforms NCAM-140 and NCAM-180 (Cavallaro and Christofori, 2001). Reduced overall levels of NCAM expression has also been shown to correlate with increased malignancy and poor prognosis in pancreatic and colorectal cancers (Fogar et al., 1997). Cavallaro et al., (2001) indicated that β 1 integrin may play a functional role in neurite outgrowth and matrix adhesion. They found that when adherent NCAM^{+/+} tumour cells were treated with neutralizing antibodies against β 1, neurite outgrowth was abrogated. A relationship between CD98 and NCAM has yet to be examined but it does seem to be a plausible concept as it could make up part of the signalling complex formed by CD98 and the β 1 integrin.

1.3.

HYPOTHESIS

There is therefore abundant evidence that CD98 is an important signalling molecule involved in cellular activation and tumorigenesis. My hypothesis is that CD98 expression leads to cellular transformation by interacting with $\beta 1$ integrins to induce a membranous signalling complex that activates intracellular signals which stimulate mitogenesis and adhesion independent growth. This investigation therefore has important implications for possible cancer therapy as this molecule appears to be present in all cancers studied so far.

1.4.

AIMS

1. To discover the role CD98 plays in transformation.
 - To examine whether this transformation is dependent on PI3-Kinase activation and serum concentration.
2. To find out whether CD98 colocalises with $\beta 1$ integrin.
 - To find out what is the nature of CD98 interaction with integrins.
 - To investigate the role of lipid rafts in the CD98/Integrin interaction.
3. To examine which part of the CD98 molecule is important in these processes.
4. To investigate the effect CD98 has on the cytoskeletal organization and microtubule network.
 - To find out whether CD98 expression induces any phenotypical changes.
5. To examine whether there are any other molecules like CD98 which have similar effects on integrins.
 - To investigate whether there are any other molecules to accompany CD98 and $\beta 1$ in a signalling complex.



CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, all chemicals were purchased from Sigma (Dorset, UK). The following reagents were obtained from Life Technologies (Paisley, UK): Dulbecco's Modified Eagles Medium (DMEM); penicillin; streptomycin; dialysed foetal calf serum (FCS); L-glutamine 30% (w/v) and non-essential amino acids.

For western blotting the following antibodies were used: anti-PKB or anti-phospho PKB (serine 473) antibody (New England Biolabs Inc., Beverly MA), anti-FAK or anti-phospho FAK antibody (pT397) (Biosource, Belgium), anti-ERK2 (C-14) or phospho-ERK1/2 (ERK-PT115) Sigma, Dorset U.K), anti-PI3-Kinase p85 N-SH3 antibody (Upstate Biotechnology, Lake Placid, USA), anti-CD98 antibody (SC-7095) (Santa Cruz Biotechnology), anti-CD56 (NCAM) antibody (Abcam Ltd., Cambridge, UK) and anti- β 1 integrin (141720 BD Transduction Labs). For flow cytometry protein A-purified 4F2 was used for identification of CD98 and CD98-chimeras containing the extracellular domain of CD98. Anti-human CD69 (clone FN50 DAKO, UK) for identification of CD69 and chimeras containing the extracellular domain of CD69. For β 1 integrin the rat clone 9EG7 was used (Pharminogen BD). For immunoprecipitation experiments the anti-human β 1 integrin K20 (DAKO, UK) was used. All anti-species specific horseradish peroxidase-conjugated antibodies were from DAKO; UK. All anti-species specific Alexa-Fluor-conjugated antibodies were purchased from Molecular Probes (The Netherlands).

Transformation competent *Escherichia coli* strain DH5 α cells were purchased from Life Technologies (Paisley, UK) and strain TOP10 cells were purchased from Invitrogen (Groningen, Netherlands);

DNA constructs

Full length human CD69 was kindly provided by Dr F Sanchez-Madrid (Madrid, Spain). The CD98 HC chimeras were made by overlap polymerase chain reaction or restriction digest and re-ligation. 98EC contains amino acids 1-81 of CD98 HC (Swiss-Prot accession number P08195), amino acids 121-183 of CD69 (Swiss-Prot accession number Q07108) and amino acids 105-529 of CD98 HC. 98ET contains amino acids 1-40 of CD69 and amino acids 82-529 of CD98 HC. 98TC contains amino acids 1-104 of CD98 HC and amino acids 62-199 of CD69. 98C contains amino acids 1-81 of CD98 HC and amino acids 41-199 of CD69. 98T contains amino acids 1-40 of CD69, 82-104 of CD98 and 62-199 of CD69. 98E contains amino acids 1-61 of CD69 and 105-529 of CD98. Δ 98 deletes amino acids 2-77, which removes the entire cytoplasmic domain of CD98 HC, maintaining the initiator methionine as well as the presumptive stop transfer sequence Val-Arg-Thr-Arg. D5 (kindly provided by Drs D Merlin and JL Madara, Atlanta, USA) deletes amino acids 1-86 which removes the entire cytoplasmic domain and the five proximal amino acids of the transmembrane domain. The above cDNAs were subcloned from pcDNA1 into the *Bam*HI and *Xho*-1-digested mammalian expression vector, pcDNA3.1 which confers neomycin resistance (Invitrogen). The subcloned plasmids were verified by sequencing. Plasmids were purified using the Qiagen Maxiplasmid kit.

2. 2Cell Culture

CHOK1 cell line was purchased from ECACC (European Collection of Cell Cultures). The CHOK1 cell line was cultured in Dulbeccos Modified Eagles Media (DMEM;Sigma), supplemented with 10% (v/v) foetal bovine serum (FBS-heat inactivated at 57°C for 1 hour), 50 IU/ml penicillin, 50 IU/ml streptomycin, 5mg/ml L-glutamine and 1% (v/v) non essential amino acids; all obtained from Life Technologies (Paisley, UK). This will be referred to as complete media.

When culturing stable cell lines expressing chimeras, G418 (antibiotic, Promega) 400 µg/ml was used to maintain expression of chimeras.

Quiescent media for CHOK1 stables was supplemented with 0.1% (v/v) heat inactivated FBS rather than 10%.

SCLC cell lines NCI-H69, NCI-H345 and NCI H510 were purchased from the American Type Tissue Culture Collection (Rockville, Maryland, USA). All SCLC cell lines were cultured in Rosewell Park Memorial Institute Medium 1640(RPMI 1640) containing 25mM Hepes supplemented with 10% (v/v) FBS (heat inactivated) and 50 IU/ml penicillin, 50 IU/ml streptomycin, and 5µg/ml L-glutamine.

Quiescent media for SCLC comprised RPMI-1640 with 25mM Hepes supplemented with 0.25% (w/v) BSA and 50 IU/ml penicillin, 50 IU/ml streptomycin, and 5µg/ml L-glutamine.

All cell lines were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C and were regularly screened for mycoplasma infection and were used at the lowest possible passage number.

2.3 Transformation of *Escherichia coli*

Frozen aliquots of strains DH5α (Life Technologies) and Top10/P3 (Invitrogen) were thawed on ice and transferred to chilled eppendorfs. In the case of Top10/P3 cells, β-mercaptoethanol 25mM final concentration was added to cell prior to addition of DNA. DNA 1-10ng was gently mixed with the cells and incubated for 30 mins on ice. Cells were then heat shocked at 42°C in a thermomixer (Eppendorf, Cambridge UK) for 45-60 seconds and returned to ice for a further 2 mins. Transformed cells were allowed to grow for 60 mins by incubating the cells in antibiotic free SOC media @ 37°C. Cells were then spread onto appropriate antibiotic selection plates. Plates were incubated at

37°C overnight in an incubator. Individual colonies were picked into LB broth containing antibiotics and grown overnight for DNA purification and restriction enzyme digest verification.

2.4 DNA Purification

Small scale (1-10 ml) DNA purifications were performed using Wizard SV Miniprep kts (Promega) as per manufacturers instructions. Large scale (100-500 ml) DNA purifications were performed using Qiagen Endotoxin Free Maxi-Prep Kit (Qiagen,Crawley UK) as per manufacturers instructions. For Low Copy plasmids the Qiagen Mini-Prep kits were used together with their low copy number plasmid protocol.

Purified DNA was checked with diagnostic restriction digests with the appropriate restriction enzymes (Promega) and were resolved on 1% Agarose (Seakem, Rockland,Maine,USA.) gels containing 0.3µg/ml ethidium bromide to enable UV visualisation.

Quantification of purified DNA was performed using a Pharmacia Biotech Ultraspec 2000 UV spectrophotometer .

2.5 Cell Transfection

Transient transfections of CHOK1 cells was undertaken using Lipofectamine™ and the Plus reagent (Invitrogen). Cells were seeded at an appropriate density to reach 50-70% confluency overnight in 60/100mm tissue culture treated dishes (Corning, High Wycombe, UK). Purified DNA was placed in 5ml BD Falcon tubes (Part No: 352034, Oxford, UK) in a volume ranging from 1-20µl. The DNA was pre-complexed with 110µl of the plus reagent and gently mixed. The Plus reagent contained Plus reagent 0.25% (v/v) diluted in DMEM containing 1% (v/v) non-essential amino acids (DMEM+AA). Following a 15 minute incubation at room temperature, the DNA was

complexed with 110µl of the Lipofectamine mixture and gently mixed. The Lipofectamine reagent contained Lipofectamine 0.25% (v/v) diluted in DMEM+AA. After a further 15 minutes incubation at room temperature, the transfection mixture was made up to a final volume of 4 ml with pre-warmed DMEM+AA and placed onto washed cells. Cells were cultured for 5 hrs at 37°C and fed with complete media (4ml). Twenty four hours after transfection, media containing DNA and lipofectamine was removed and replaced with fresh complete media. For experiments where protein kinase activities were to be assessed, the transfection media was replaced with quiescent media after 24 hrs.

2.5.1 Production of stable cell lines

For each plasmid DNA to be stably expressed, CHOK1 cells were transfected in 100mm dishes using 3µg of plasmid DNA. Forty eight hours post transient transfection, CHO-K1 complete media was supplemented with G418 at 800µg/ml (stable selection media). Stable selection media was replaced frequently as untransfected cells began to die and selected cells grew back to confluency. Once the 100mm plates were fully confluent, cells were trypsinised, washed and resuspended in PBS (w/o ca and mg). The single cell suspension was seeded into 96-well plates at a density of approximately one cell/well. Cells were incubated at 37°C until clonal growth was observed in the wells (colour change). Cells were trypsinised from the wells and plated out in duplicate wells (of 6-well plates) and grown to confluency in selection media. Clones showing equivalent wild type, mutated or truncated human CD98 expression by SDS page and western blot analysis or by intracellular FACS staining were selected for this study.

2.6 Assessment of protein concentration

Protein concentrations were quantified using a BCA protein assay (Pierce, IL,USA). A protein standard curve was generated using bovine serum albumin (Sigma) at a range of 0.1-0.5mg/ml diluted in 1:10 lysis buffer. Samples were diluted 1 in 10 in dH₂O and 10µl was incubated with 200µl BCA reagent (30mins, 37°C) in 96 well plates. Protein

concentration was determined using an automated plate reader (MRX microplate reader, Dynatech, Chantilly, VA).

2.7 SDS-PAGE and Western blotting

2.7.1 Cell lysis

Transfected cells were washed once with ice cold PBS and lysed at 4°C in an appropriate amount of lysis buffer. Lysates were cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C, assessed for protein concentration (described above) and solubilised in 4x sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 99°C for 5 minutes. Samples were analysed immediately or stored at -20°C for future analysis.

Buffer

50mM Hepes (Na Salt), 0.3M NaCl, 1.5mM MgCl₂, 1.2mM EDTA, 0.5% Triton X-100, 20mM β-glycerophosphate, 100mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium vanadate, 0.5mM dithiothreitol (DTT). One completeTM protease inhibitor tablet (Boehringer Mannheim, Lewes, UK) was added per 50 ml of lysis buffer.

2.7.2 SDS-PAGE and Western Immunoblotting

Samples were cooled and equal amounts of protein (10-100μg) were resolved on SDS polyacrylamide gels using a vertical electrophoresis tank Biorad protean 3 system (Biorad, Hemel Hempstead, UK) and (ATTO corp., Japan). Samples were electrophoresed at 100-150 Volts using SDS-Tris-Glycine electrophoresis buffer for 1-2 hours beside pre-stained molecular weight markers (Life Technologies, Paisley, UK). For optimal separation, 8% gels were used for 60-120 kDa proteins, 10% for 40-70 kDa proteins and 12% gels for 10-40 kDa proteins.

Proteins were transferred onto Hybond C nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, UK). in a methanol based transfer buffer at 400

milliamps for 1hr using a Mini Protean II blotting tank (Biorad, UK). Equal protein loading was confirmed by incubating blots for 5 minutes in Ponceau S (Sigma), which allows visualisation of protein bands. Non-specific protein binding sites on the membranes were blocked by incubation in TBS-Tween 20 containing 5% non-fat dried milk powder overnight at 4°C. Membranes were washed in TBS-Tween 20 (3 x 30 second vigorous washes and 3 x 10 minute washes) before species appropriate horseradish peroxidase (HRP) conjugated secondary antibody (DAKO, Ely, UK.) (diluted in washing buffer) was added for 1 hr.

Finally membranes were rewashed as above and immunoreactive bands were identified using enhanced chemiluminescence, (ECL) (Amersham Pharmacia Biotech, Amersham, UK.) as per manufacturers instructions.

Buffers

4x SDS-PAGE sample buffer

50mM Tris-HCl, 10% (v/v) glycerol, 2% (v/v) SDS, 0.1% (v/v) bromophenol blue and 10% (v/v) β -mercaptoethanol.

Gel electrophoresis buffer

5mM Tris Base (pH 8.3), 25mM glycine and 0.01% (v/v) SDS.

Transfer buffer

210mM glycine, 24.7mM Tris Base and 20% methanol (v/v) methanol.

TBS-Tween 20 Wash buffer

TBS containing 0.2% (v/v) Tween 20 (Sigma).

SDS polyacrylamide gels

Separating Gel: 0.375 M Tris Base (pH 8.8), 0.1% (v/v) SDS, 8-12 % (v/v) acrylamide (Life Technologies, Paisley, UK.), 0.1% (v/v) ammonium persulphate and 0.08% (v/v) TEMED.

Stacking Gel: 0.13 M Tris Base (pH 8.8), 0.15% (v/v) SDS, 4.6% (v/v) acrylamide, 0.13% (v/v) ammonium persulphate and 0.1% (v/v) TEMED.

2.8 Flow Cytometry

Aliquots of 5×10^5 cells were washed and resuspended in 100ul PBS containing 1µg 4F2 (for CD98 and chimeras containing the extracellular portion of CD98) or 1µg anti-CD69 antibody (for CD69 and chimeras containing the extracellular portion of CD69). Cells were incubated for 30 minutes at room temperature followed by two washes with PBS. Samples were then incubated with species specific appropriate FITC-conjugated secondary antibody (1:50) (DAKO, UK.) for 30 minutes at 4°C and again washed twice in PBS. Samples were finally resuspended in PBS and analysed by flow cytometry using FACS Calibur™ (Becton Dickinson). Control IgG2a and IgG1 antibodies for 4F2 and CD69 respectively were also used.

2.9 Liquid growth Assay

Stably expressing CHOK1 cells were seeded into 100cm³ plate and left to grow for 10 days in a humidified atmosphere of 5% CO₂/95% air at 37°C. Plates of cells were then removed at various time points and trypsinised and counted. Cell number was determined using a Coulter Counter (Coulter Electronics, Luton, UK).

2.10 Immunofluorescence

Transfected cells or stable cell lines were grown on sterile glass cover slips (22 x 22 mm, Fisher, Loughborough, UK) placed within 6-well tissue culture plates (Corning, High Wycombe, UK), and left overnight in complete medium at 37°C. After 2 days of growth, the media was removed and the cells were washed with PBS x 2.

2.10.1 Indirect Immunofluorescence- Formaldehyde Fixation

Cells were fixed using 2mls of 3% Paraformaldehyde solution (PFA) for 20 minutes at room temperature. The PFA was then removed and the aldehyde groups were quenched by incubating the cells with a solution of 50mM NH₄Cl/ CMF-PBS for 10 minutes at room temperature. After further washing with TBS, non-specific binding sites were blocked using 1-2mls FSG(0.2% Fish skin gelatin) for 30-60 minutes or overnight at 4°C.

2.10.2 Methanol (protein precipitation) Fixation

Coverslips were immersed in -20°C 100% methanol for 10 minutes. Cells were then washed 3 x for 5 minutes in room temperature TBS. Cells were then quenched in fresh 0.1% sodium borohydride in TBS for 5 minutes. Non-specific binding sites were blocked with 1% BSA for 1hr with TBS.

2.10.3 50:50 Methanol:Acetone Fixation

Coverslips were immersed in -20°C 50:50 Methanol:Acetone for 30 seconds. Cells were then washed 3x for 5 minutes in room temperature TBS. Non-specific binding sites were blocked using 1-2mls FSG(0.2% Fish skin gelatin) for 30-60 minutes or overnight at 4°C.

2.10.4 Triton Fixation : Used for cytoskeletal components.

Cells were fixed in 3% PFA for 3-5minutes at room temperature and then washed x 2 with PBS. Cells were then permeabilised with 0.1% TritonX100 for 3 minutes. Cells

were then washed in 1x PBS + 0.05% Triton X 100 (PBSTX). Non-specific binding sites were blocked with an incubation in FSG for 1hr at room temperature.

After each fixation cells were probed with appropriate primary antibodies (Table 2.10.5) diluted in 0.2% FSG for a variety of different times either at room temperature or at 4°C. Cells were washed with PBS/TBS/PBSTX (3 x 2 minute washes) before species appropriate Alexa-Fluor-conjugated antibodies (Molecular Probes, The Netherlands) was added, diluted in 0.2% FSG for 30 minutes on ice and in the dark. Cover slips were removed from the tissue culture plates, washed in dH₂O and allowed to dry. Coverslips were mounted on slides using Mowiol.

All cells were visualised using a Leica TCS NT confocal microscope (Leica Microsystems, Heidelberg GmbH) and images captured and analysed using Leica Confocal Software - Lite Version (Leica Microsystems, Heidelberg GmbH).

Primary antibody/drug	Concentration /dilution	Incubation time and conditions	Secondary antibody	Concentration /dilution
IgG2a antibody	20µg/ml	20minutes at room temperature.	Alexa488/568-conjugated goat anti-mouse IgG	1:100
IgG1	20µg/ml	20minutes at room temperature.	Alexa488/568-conjugated goat anti-mouse IgG	1:100
4F2 (anti CD98)	20µg/ml	1hr at 4oC	Alexa488-conjugated goat anti-mouse IgG	1:100
9EG7 (anti β1)	1:50	Overnight at 4oC	Alexa568-conjugated goat anti-rat IgG	1:100
FN50-FITC (anti CD69)	1:25	20minutes at room temperature.	Alexa488-conjugated goat anti-mouse IgG	1:100
Rhodamine-conjugated Phalloidin	1:50	20minutes at room temperature.		
Vinculin (monoclonal antibody)	1:400	20minutes at room temperature.	Alexa488-conjugated goat anti-mouse IgG	1:100
Phospho FAK	1:50	20minutes at room temperature.	Alexa488-conjugated goat anti-rabbite IgG	1:100
Tubulin (monoclonal antibody)	1:200	20minutes at room temperature.	Alexa488-conjugated goat anti-mouse IgG	1:100
NCAM	20µg/ml	20 minutes at room temperature	Alexa488-conjugated goat anti-mouse IgG	1:100
Sc7094 (anti CD98) (polyclonal)	1:50	20 minutes at room temperature	Alexa568-conjugated donkey anti-goat IgG	1:100
Bek (FGFR2)	1:50	20 minutes at room temperature	Alexa568-conjugated goat anti-rabbit	1:100
CD71 (transferrin receptor)	1:50	20 minutes at room temperature	Alexa488-conjugated goat anti-rabbit	1:100

Table 4 Antibodies used in immunofluorescence studies

2.11 Manipulation of plasma membrane cholesterol content using Methyl- β -cyclodextrin

Cells required for immunofluorescence were left attached to coverslips inside 6-well plates with 2ml of quiescent media.

Cells were treated with PBS containing M β CD (Sigma) to a final concentration of 1% and incubated for 1 hour at 37°C. Following incubation, M β CD-containing media was removed and the cells were resuspended in 2ml quiescent media. For experiments requiring cholesterol replenishment, 0.5mM cholesterol/ml was added to quiescent media for 1 hour at 37°C in the form of cholesterol-M β CD inclusion complexes. Cholesterol-M β CD inclusion complexes were produced as described by Klein *et al.*, (1995). In brief, a solution of 30mg cholesterol (Sigma) in 400 μ l of 2:1 (v/v) methanol/chloroform was added drop-wise to a stirred solution of 1g M β CD in 11ml PBS prewarmed in an 80°C waterbath. The solution was stirred at 80°C until a clear solution resulted. The cholesterol-M β CD inclusion complexes were used immediately or aliquoted and lyophilised. For convenience, 375 μ l (5 x 69.4 μ l) aliquots of the cholesterol-M β CD inclusion complex solution were lyophilised. For use, the individual aliquots could be resuspended in 5ml PBS to provide 5 x 1ml treatments (0.5mM cholesterol).

Following treatment, cells were washed in PBS. Cells were then labelled with appropriate antibodies or fixed for immunofluorescence as mentioned in section?

2.12 Anchorage-Independent Growth Assays (Colony assays)

CHOK1 cells (2×10^3 /ml) were suspended in 0.3% (w/v) agarose in DMEM containing 1% FCS unless otherwise indicated. The cells were layered over a solid base of 0.5% (w/v) agarose in DMEM in 6 well culture dishes. Cultures were incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C. After 6 days, colonies greater than 4 cells were counted using light microscope.

A 50:50 mixture of Seakem agarose (Cambrex Bioproducts, Maine): low melting point agarose (Sigma), was used to prepare 5% and 3% (w/v) (10x) stock solutions of agarose in sterile water, in autoclaved bottles. Solutions were boiled to dissolve the agarose and stored at room temperature. On the day of the assay, stock solutions were melted and maintained at 45°C in a water bath. The 0.5% agarose layer was prepared by diluting the 5% stock solution in warmed growth medium (i.e., DMEM + required serum concentration); 2ml were added to each well of a 6 well plate.

Once the bottom layer had set, a single cell suspension containing 1×10^3 cells per ml of growth medium (i.e., DMEM + required serum concentration + any required antibiotics) was warmed and the melted 3% agarose stock was added to a final concentration of 0.3%. The cell and agarose suspension was mixed gently by pipetting to resuspend the cells uniformly, then 2ml per well was added to the hardened 0.5% agar base layer and left to set. Plates were covered with tinfoil, to prevent drying-out, and incubated at 37°C in a humidified 10% CO₂ environment. On day 6, the number of colonies from five random fields was counted for each replicate.

Percentage cloning efficiency was calculated as follows:

$$(((Mean / 5^*) \times 160^f) / 2000^§) \times 100.$$

Mean Total colonies counted for each sample divided by the number of replicates (in this case, n=3).

* Number of fields viewed per well.

f (Well area/ view area). Multiplying by this figure allows an approximation of the total number of colonies per well to be made.

§ Number of cells seeded

For colony assays using SCLC cells, cells were washed and resuspended in quiescent media before being disaggregated using a cell strainer into a single cell suspension. 1×10^3 cells /ml were added onto the top of the 0.5% (w/v) agarose layer in a 24 well plate.

A 0.3% (w/v) agarose layer was then layered on to the top of the cells. After 3 weeks SCLC colonies were stained using 5mg/ml MTT and then scanned in order to make comparisons between treatments.

2.13 PI3-Kinase Activity Assay

Cultures of stably expressing CHOK1 cells were washed and resuspended in quiescent media and incubated at 37°C for 24 hours. The following day cells were washed with PBS and then treated with either :

- 100nM Wortmannin for 20 minutes at 37°C

- 20µg/ml 4F2 for 10 minutes at 37°C

- 10% FCS for 10 minutes at 37°C

The media was removed with an aspirator and the plates were washed with cold PBS. The plates were then transferred to an ice bath. 1ml of ice cold lysis buffer was added to the plates. The plates were then scraped and the cells were put into cold 2 ml eppendorfs.

Ice cold Lysis Buffer:

50mM HEPES (pH 7.4), 150mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5mM MgCl₂, 1mM EGTA, 100mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 0.5mM dithiothreitol and 1 protease inhibitor tablet to 50ml buffer.

The cells were then vortexed for 15 minutes at 4°C and then clarified by centrifugation at 13,000 rpm for 10 minutes at 4°C. Protein concentration was equilibrated by performing a BCA protein assay. For each assay point 1µg of p85-SH3 PI3-Kinase antibody was added and incubated overnight at 4°C.

30µl of goat-anti-mouse IgG agarose beads in lysis buffer were added for an additional 2 hours. The beads were then pelleted and washed twice in lysis buffer, once with a high

salt buffer containing 100mM TrisHCl and 500mM LiCl (pH 7.6) and twice with a solution containing 200mM HEPES, 40mM MgCl₂ and 600mM NaCl (pH 7.4).

PI3-Kinase assays were performed using phosphatidylinositol as a substrate in a final volume of 200μl containing : 50mM HEPES (pH7.4), 10mM MgCl₂, 150mM NaCl, [γ^{32} P] ATP (5uCi, 500 Ci/mmol) 50uM ATP and 0.5mg/ml sonicated phosphatidylinositol: phosphatidylserine (3:1 w/v).

Reactions were carried out for 10 minutes at 37°C and terminated by addition of chloroform:methanol: 0.1M HCl (40:80:1 v/v/v; 750μl), and phase partition was achieved by the addition of chloroform (250μl) and 0.1M HCl (250μl). After centrifugation, the lower phase was washed twice with synthetic upper phase (methanol:1M HCl: chloroform, 48:47:3 v/v/v), dried and lipids were separated using thin layer chromatography (TLC).

The TLC running solution consisted of methanol:chloroform:ammonia solution:water, in the proportions 100/70/15/25 (v/v/v/v). 3' phosphorylated lipids were identified by autoradiography using Kodak "X-OMAT" Film and radioactivity was quantified by liquid scintillation counting using Flo-Scint IV.

2.14 Mass measurement of PtdIns(3,4,5) P₃.

This assay requires:

- a) GAP1^{IP4BP}
- b) Samples
- c) Ins(1,3,4,5) P₄ standards
- d) [³²P] Ins(1,3,4,5)P₄ label
- e) Wash buffer
- f) Assay buffer

a) Cultures of 2 x 5ml of GAP^{IP4} in LB Broth with ampicillin and kanomycin (1:1000) were grown overnight at 37°C. The overnight culture (2.5ml) was then added to 500mls of LB Broth with ampicillin and kanomycin, and incubated for 2 hrs at 37°C with mixing. IPTG (0.5mM) was then added and the culture was incubated for a further 5 hrs at 37°C. The culture was then centrifuged at 4000rpm, the supernatant discarded and the pellet resuspended in 9mls of cold *E.Coli* lysis buffer.

E.Coli lysis buffer:

53mM Na₂HPO₄, 3mM NaH₂PO₄, 4mM NaCl, 1mM EDTA, 1mM EGTA,

1mM β-mercatoethanol

Lysozyme(2mg/ml) was then added and kept on ice for 30 minutes. This was then sonicated on ice for 5 x 30 second bursts. 2% v/v Triton X-100 was then added and kept on ice for 30 minutes. This solution was then centrifuged for 30 minutes and the supernatant collected and incubated with prepared glutathione agarose beads for 2hrs (0.2g of glutathione agarose was swelled with 12.5mls of H₂O for 2hrs, then washed 4 x with the *E.Coli* lysis buffer). This mixture was then washed 2x with 10 mls of cold lysis

buffer and 2 x with high salt buffer, then resuspended in 20mls of high salt buffer and glycerol. This solution was then mixed, aliquoted and kept at -20°C .

b) 2×10^6 cells were seeded into 10cm^3 tissue culture plates and then when cells were at ~ 60-70% confluency were resuspended in quiescent media overnight at 37°C .

Stage 1

The cells were then treated with agonists or antagonists if needed, medium was aspirated and then washed with PBS. The cells were then fixed with 1ml of 10% TCA for 10 minutes on ice, scraped off and collected in eppendorfs. The cell lysates were centrifuged at 13,000rpm for 2 minutes and the pellets were then washed with 5% TCA/1mM EDTA. Lipids were extracted using 750 μl of (40:80:1 v/v/v) $\text{CHCl}_3/\text{MeOH}/\text{conc HCl}$ and incubated on ice for 20 minutes. The phases were then split by the addition of 250 μl of CHCl_3 and 450 μl of 0.1M HCl. The lower phase, obtained after a minute centrifugation at 13,000 rpm was transferred to a screw cap tube and the upper phase was re-extracted once with 450 μl of the synthetic lower phase. The lower phases were pooled and dried down.

Stage 2

Alkaline hydrolysis of dried lipids was carried out by vortexing in 50 μl of 1M KOH and incubated at 100°C for 30 minutes. Neutralisation was then carried out by adding 50 μl of 1M Acetic acid and the fatty acids removed by 2 extractions with 0.5mls of water saturated butanol/petroleum ether/ethyl acetate (20:4:1 v/v/v). The resulting water soluble fractions were dried down in a speedivac for 3 hours and resuspended in 110 μl of dilute acetic acid to a final pH of 5.0.

c) **The Ins(1,3,4,5)P₄ standards** containing 0.5M KOH/acetic acid were prepared at the following concentrations: 8×10^{-6} M, 24×10^{-7} M, 8×10^{-7} M, 24×10^{-8} M, 8×10^{-8} M, 24×10^{-9} M, 8×10^{-9} M, 24×10^{-10} M, 8×10^{-10} M, 24×10^{-11} M, 8×10^{-11} M.

d) 0.001 μ Ci [³²P]Ins(1,3,4,5)P₄ label was used per assay point.

e) **Wash buffer:**

25mM Sodium acetate, 25mM KH₂PO₄, 1mM EDTA, 5mM NaHCO₃, pH5 with acetic acid

f) **Assay buffer:**

0.1M Sodium acetate, 0.1M KH₂PO₄, 4mM EDTA, pH5 with acetic acid

Stage 3

The standards were prepared as follows:

10 μ l of standard

40 μ l H₂O

50 μ l 1M Kac pH5

The samples were made up to a final volume of 400 μ l and comprised of:

100 μ l of sample or standard

100 μ l of assay buffer

100 μ l [³²P]Ins(1,3,4,5)P₄ (0.001 μ Ci in 100 μ l of H₂O)

100 μ l GAP^{IP4BP}

On addition of the binding protein the samples were vortexed and incubated on ice for 30 minutes, with occasional vortexing. Filtration of bound [³²P]Ins(1,3,4,5)P₄ from unbound was achieved by filtration through GF/C filters (Whatman;Maidstone,UK). The samples were filtered onto pre-wetted filters and immediately rinsed x 2 with 10mls of ice cold wash buffer. The filters were then allowed to air-dry and transferred to

scintillation vials containing 3 mls of “Floscint V” (Packard Biosciences; Groningen, Netherlands) and the radioactivity counted in a liquid scintillation counter.

2.15 Immunoprecipitation

Confluent cultures of CHOK1 cells from 100 mm³ plates were quiesced overnight in 0.1% FBS media and washed in PBS. SCLC cells were quiesced overnight, centrifuged at 1000 rpm for 4 minutes then the resulting pellet was washed with PBS. All cells were lysed at 4°C in lysis buffer comprising 20 mM Hepes pH 7.4, 1% CHAPS, 150 mM NaCl, 2 mM MgCl₂, 1 mM MnCl₂, 0.5 mM CaCl₂ and protease inhibitors (EDTA-free protease inhibitor cocktail, Roche, Germany) or the MAPK lysis buffer as mentioned in 2.7.1 and clarified by centrifugation at 13,000 x g for 10 min at 4°C. The lysate (1mg/ml protein) was incubated with 2 µg immunoprecipitating antibody overnight at 4°C. Immune complexes were captured using 15 µl Protein-G agarose and washed x3 with lysis buffer. Following elution with Nu-PAGE buffer associated proteins were resolved on 4-12% gradient gels. The proteins were transferred to nitrocellulose membranes, blocked using 3% (w/v) albumin in TBS-Tween (20 mM Tris-HCL (pH 7.4), 150 mM NaCl, and 0.02% (v/v) Tween 20) for one hour at room temperature and then incubated with primary antibody overnight at 4°C. Species appropriate horseradish peroxidase-conjugated antibodies (DAKO, UK) were used for secondary labeling. Immunoreactive bands were identified using enhanced chemiluminescence (Amersham, UK) according to the manufacturer’s instructions.

2.16 Cell adhesion assay

SCLC cells were quiesced for 2 hours at 37°C in RPMI containing 0.25% BSA. During this time a 96 well plate was coated with various ligands. Negative controls were left

empty and positive controls were coated with 100µg/ml poly-L-lysine. 100µl of ligand was added to each well and incubated for an hour at 37°C. The plate was then washed with PBS and each well was blocked using 2% BSA for 1 hour at 37°C, to prevent non specific binding. Quiesced SCLC cells were then pelleted and counted. 100µl of cells were then added at 1×10^5 per well and then incubated at 37°C. After an hour the cells are removed from the wells, washed x 2, using 100µl of PBS and then fixed for 20 mins using 100µl 3% PFA (Paraformaldehyde). The fixative was then removed and left to air dry overnight. Once dry, methylene blue was added to the wells and left for 30 minutes at room temperature. After several washes in water, the plate was left to dry again. After drying 100µl of 0.1M HCl was added to the wells and once the colour in the wells became uniform, the absorbance is then measured at 630nm.

The percentage of adhesion is then calculated compared to the controls. It is calculated as shown below:-

$$\text{adhesion} = \frac{\text{Ligand} - \text{negative control}}{\text{Positive control} - \text{negative control}} \times 100\%$$

2.17 Production of NCAM Fusion Protein (NCAM-FC)

CHOK1 cells at ~50-60% confluency were transfected as mentioned in section 2.5 with the NCAM-FC plasmid. The chimaeric protein was allowed to accumulate in the medium for 6 days. The medium was then collected and spun down to remove any cell debris and purified with protein A sepharose. Protein A sepharose beads were washed x 2 in PBS and then added to the supernatant and incubate overnight at 4°C on a roller. The mixture was then centrifuged at 1000 rpm for 4 minutes and the supernatant removed, the pellet washed and then transferred with 1ml of PBS to an eppendorf. This was then centrifuged at 13,000rpm for 1 minute and the supernatant removed. To elute the protein 100mM Glycine HCl (pH 2.7) was added and left on ice for 5 minutes, spun down again at 13,000rpm and then the resulting supernatant was then collected and

added to 1MTris HCl (pH 9) to neutralise the glycine. Ten fractions were collected. In order to determine the location of the protein, fractions were run on an SDS-PAGE gel and the gel was stained with Comassie blue. The fractions containing the protein were then pooled, dialysed in PBS overnight at 4°C and the protein quantified using the BCA protein assay.

CHAPTER 3

THE CLONING AND CONSTRUCTION OF CD98 AND CHIMERIC STABLE CELL LINES.

3.0 INTRODUCTION

The aim of this present study was to investigate the structure/function relationship between CD98, integrin association, intracellular signalling and transformation. In 2000, Fenczik et al., indicated that the cytoplasmic and transmembrane domains of the CD98HC are important for integrin function and the extracellular domain was required for amino acid transport. In order to elucidate which regions in the CD98 molecule are functionally important, chimeras of CD98 were obtained from Csilla Fenczik (Scripps). These chimeras varied in domains of CD98 and another molecule CD69.

CD69 is another type II transmembrane heterodimeric glycoprotein with a C-type lectin binding domain which acted as a control against CD98 (Natarajan et al., 2000). CD69 is a molecule with a wide cellular distribution and when crosslinked intracellular signals are induced. This induction of signals suggests a role for the CD69 receptor in the biology of hematopoietic cells. Similar to the molecule CD98, it has been reported that CD69 may be involved in the pathogenesis of diseases such as rheumatoid arthritis, chronic inflammatory liver diseases, mild asthma and AIDS (Marzio et al., 1999). As originally seen with CD98, CD69 once expressed acts as a co-stimulatory molecule for T cell activation and proliferation (Ziegler et al., 1994).

The chimeras of CD98 and CD69 were made by overlap polymerase chain reaction or restriction digest and re-ligation. All of these constructs were originally cloned into pcDNA1 vectors and are described in detail in MATERIALS AND METHODS and shown in Figure 3. These chimeras were then subcloned into pcDNA

3.1 which contains a G418 selection marker and stable cell lines of each were produced. All of this work was carried out in collaboration with Neil Henderson, another PhD student in my laboratory.

The chimeras vary in domains of CD98 and CD69 and are described briefly down below:

1. 4F2TM:- consisted of the extracellular domain of CD69 and the transmembrane and cytoplasmic domains of CD98.
2. 4F269:- consisted of the extracellular and transmembrane domain of CD69 and the cytoplasmic domain of CD98.
3. 69CYTO:- consisted of the extracellular and transmembrane domain of CD98 and the cytoplasmic domain of CD69.
4. 69TM:- consisted of the extracellular domain of the extracellular domain of CD98, the transmembrane domain of CD69 and the cytoplasmic domain of CD98.
5. Δ 98:- consisted of the extracellular and transmembrane domains of CD98, but had the cytoplasmic domain deleted.
6. D5:- consisted of the extracellular domain of CD98 and a truncated CD98 transmembrane domain of CD98.

Two extra chimeras (98EX and 98TM), containing the significant regions in the CD98 molecule were designed and constructed by Neil Henderson in order to critically assess how important the transmembrane and extracellular regions of CD98 were in the structure/function relationships examined in this study.

7. 98TM:- consisted of the extracellular domain of CD69, the transmembrane domain of CD98 and the cytoplasmic domain of CD69.
8. 98 EX:- consisted of the extracellular domain of CD98 and the transmembrane and cytoplasmic domains of CD69.

3.1 Transformation of chimeras.

Initially, chimera DNA was transformed into TOP10/P3 *E.coli* in order to produce DNA for CHOK1 cell transfection. Transformation was carried out as described in MATERIALS AND METHODS section 2.3. In brief, 1µl of each plasmid was incubated with 50µl of competent TOP10/P3 cells on ice for 30 minutes. The cells were then heat shocked for 60 seconds at 42°C and 450µl SOC media was added. The cells were then shaken in a thermomixer for 1 hour at 37°C and then centrifuged at 4000 rpm for 4 minutes. The pellet was then resuspended and spread onto selective agar plates and left overnight at 37°C. Single colonies were transferred to LB broth containing the antibiotics ampicillin and grown overnight at 37°C.

Minipreps and maxipreps (Qiagen) were then carried out to purify the DNA, as described in MATERIALS AND METHODS, Section 2.4. The purified DNA was then verified for content with diagnostic restriction digests as shown in figure 3.1.1. The digests show 69TM and 69CYTO digested with both Bam H1 and Xho 1 or just with Bam H1 restriction enzymes and confirm that the cloning of the two cDNA's, 69TM and 69Cyto had been achieved. Figures 3.1.2 and 3.1.3 show restriction maps of vectors 69TM and 69CYTO and 4F269 and 4F2TM.

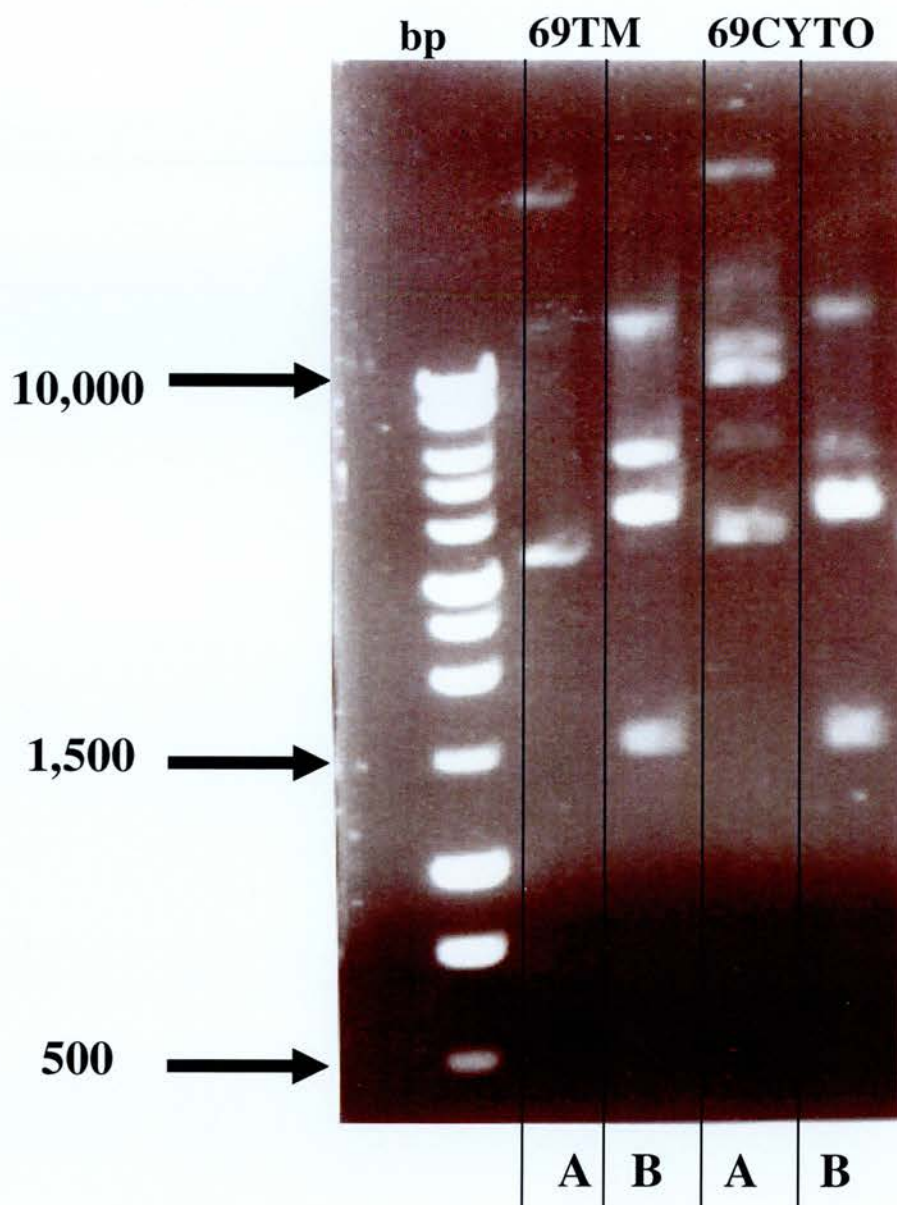
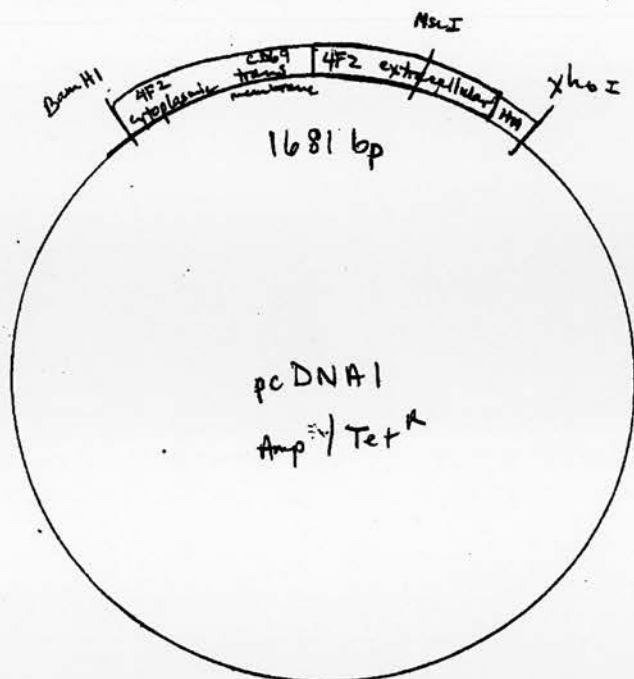


Figure 3.1.1 Verification of DNA content of 69TM and 69CYTO.

Verification of DNA content was carried out using restriction digests. The digests were then run on a 1% agarose gel containing ethidium bromide allowing visualisation using UV light. This figure shows 69TM and 69 CYTO fragments obtained from digests with A) Bam H1 alone or B) Bam H1 and Xho 1 restriction enzymes.

a)



b)

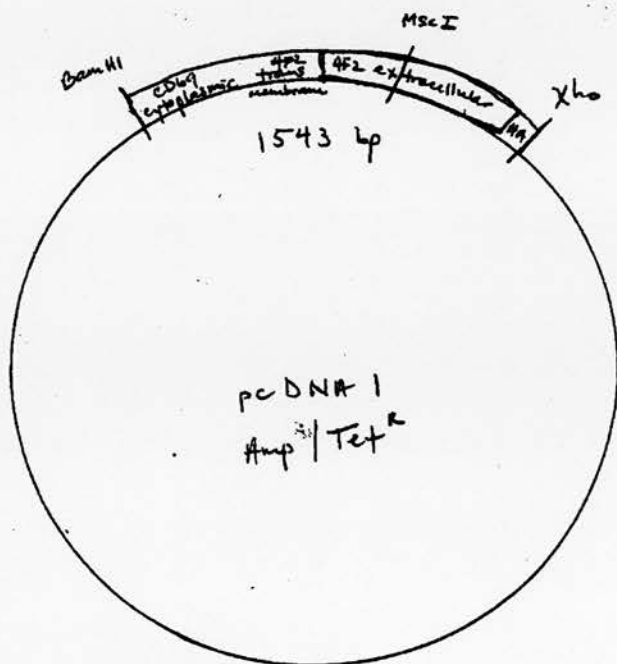
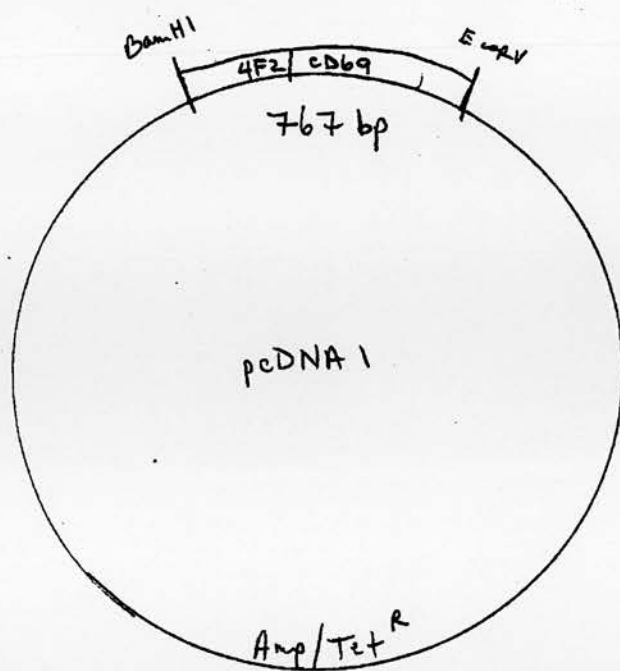


Figure 3.1.2 a) 69TM and b) 69CYTO in the vector pcDNA1

a)



b)

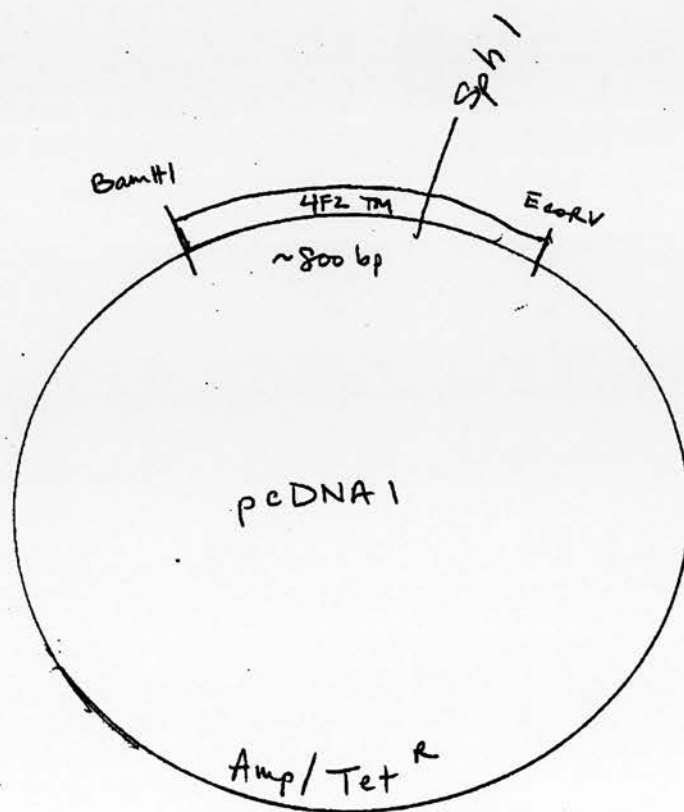


Figure 3.1.3. a) 4F269 and b) 4F2TM in the vector pcDNA1

3.2 Transfection

Having verified the DNA content, CHOK1 cells were transfected with the chimeric DNA using lipofectamine plus, described in MATERIALS AND METHODS, Section 2.5. The transfection efficiency of each chimera was analysed using flow cytometry or expression was checked by western blotting. An example of this step is shown in figure 3.2.1. In figure 3.2.1, panel A, the western blot shows successful transfection of 69TM and 69CYTO and panel B shows the results from FACS analysis carried out on another chimera 4F269. The FACS analysis shows a variably enhanced signal of FITC/PE binding which indicates that a good transfection efficiency of 4F269 was achieved.

It became evident after several different experiments that in order to study events such as transformation and integrin association, stable cell lines of each chimera were required. Chimeras were subcloned into pcDNA 3.1 and stable cell lines were produced under neomycin selective pressure. Single cell clones were isolated, propagated and screened by FACS analysis and western blotting.

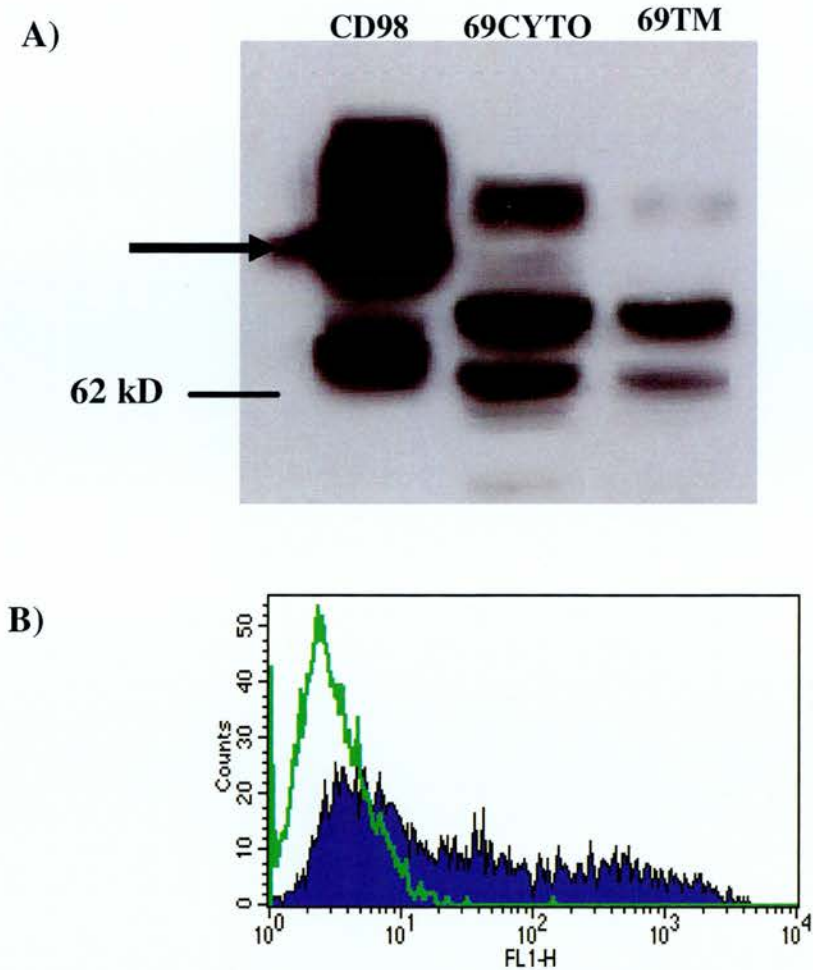


Figure 3.2.1 Analyses of transfection efficiency and expression of chimeras.

A) CHOK1 cells were transiently transfected with either of the two chimeras, 69TM or 69CYTO which are HA tagged. As a control CD98 was also transfected alone into cells. The cells were lysed after 24hrs and run on an SDS-PAGE gel, transferred to nitrocellulose membrane and probed for HA. This blot shows successful transfection of the HA-tagged chimeras. The arrow indicates the positive control CD98HC.

B) Transient transfection of CHOK1 cells with 4F2-69 cDNA. The black shading represents FITC/PE binding in transfected cells; representing expressed protein on the surface.

3.3 Subcloning

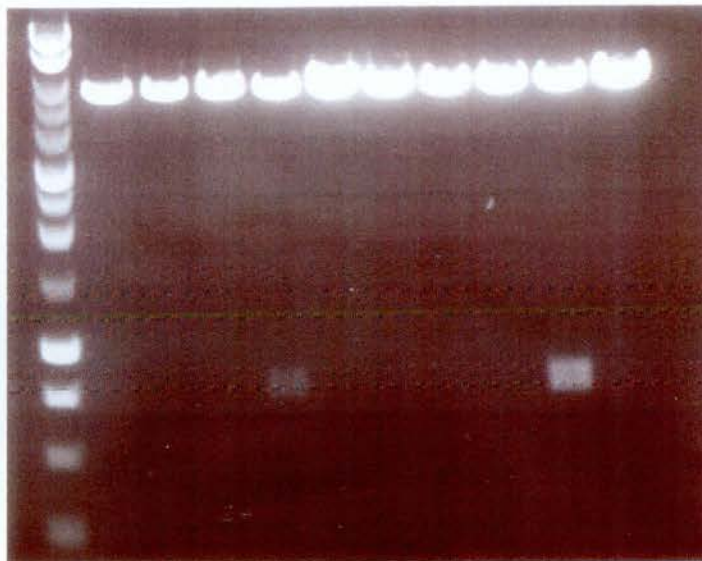
pcDNA1 (5µg) with the chimera insert and 2µg of pcDNA 3.1 were digested using Bam H1 and Xho 1 restriction enzymes for 2-3 hours at 37°C, heat inactivated at 80°C for 15 minutes and then left to cool down. The inserts and the vectors were then run on a 1% agarose gel containing ethidium bromide. The bands were visualized using UV light and the bands containing the inserts and the vectors were removed. The DNA was then purified using a Qiagen gene extraction kit, eluted and quantified using a spectrophotometer.

Ligation was then performed using the calculation below:

$$\text{ng insert} = \text{amount of vector (100ng)} \times \frac{\text{insert size (kb)}}{\text{vector size (kb)}} \times 3 \text{ (ratio)}$$

The ligation mixture was incubated at room temperature for 2-3 hours. Transformation of the ligation mix was performed as described in section 3.1 but this time it was carried out using DH5 alpha competent cells. After incubation overnight at 37°C mini-preps were carried out and the samples were run on gels and visualized using UV light. The mini-preps carried out are shown in figure 3.3.1 and 3.3.2. Maxi-preps were carried out on the colonies selected and diagnostic restriction digests were performed. Digest gels are shown in figure 3.3.3. Having confirmed an appropriately sized insert following restriction digest, each clone was further verified by DNA sequencing.

A)
4F2TM



B)
4F269

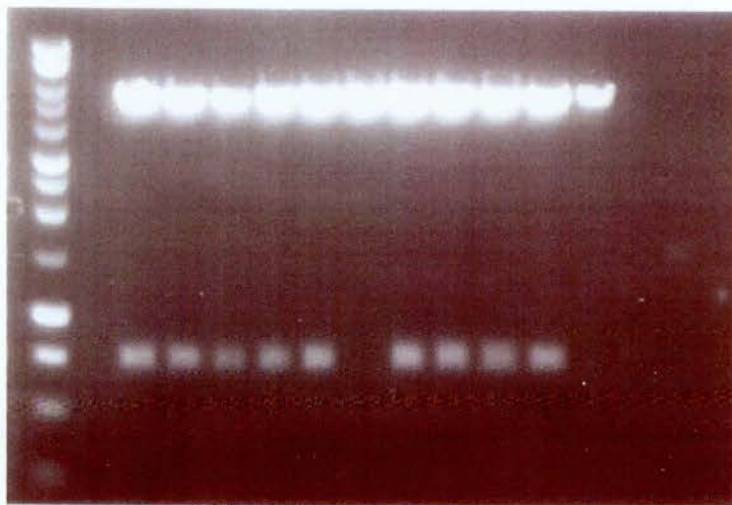


Figure 3.3.1. 4F2TM and 4F269 subcloned from pcDNA 1 to pcDNA 3.1.

A) Mini-prep showing insert in 2 colonies in subclone 4F2TM. Insert size ~ 800 base pairs. Colony 9 was selected for maxi prep. B) Mini-prep showing insert in 9 colonies in subclone 4F269. Insert size 767 base pairs. Colony 1 was selected for maxi prep.

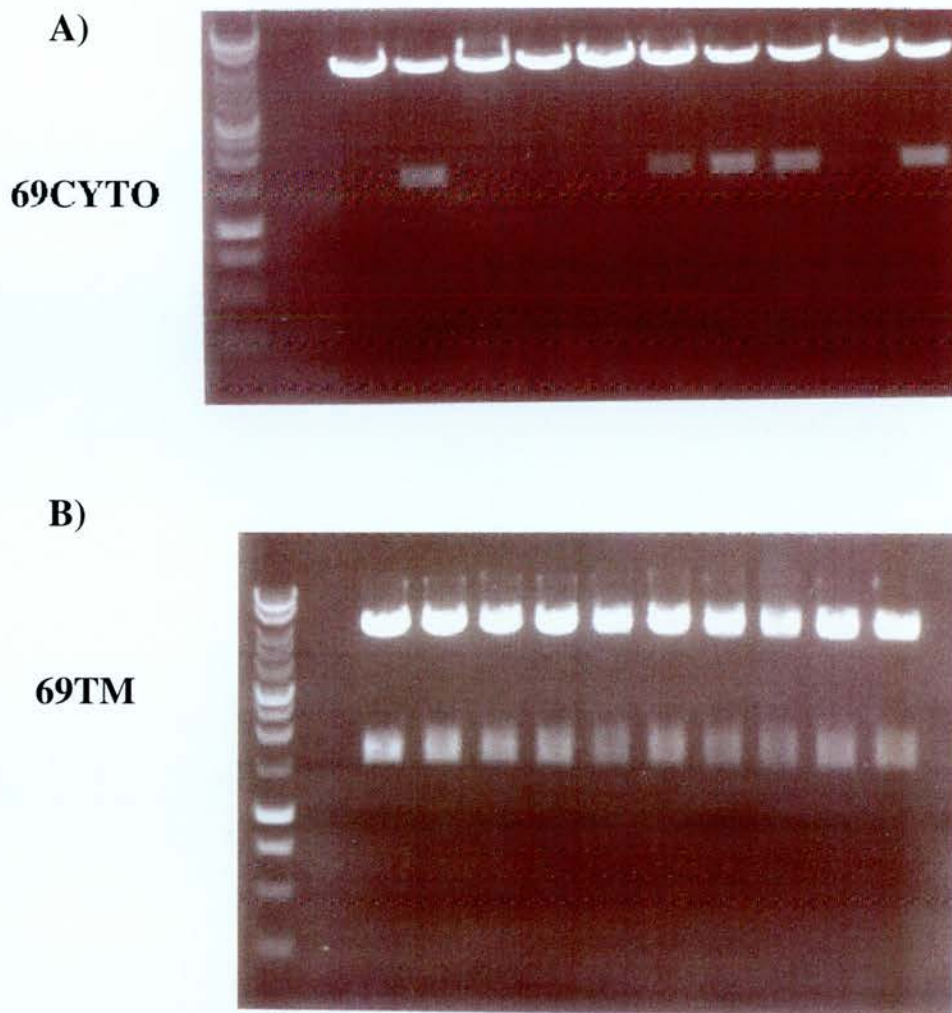
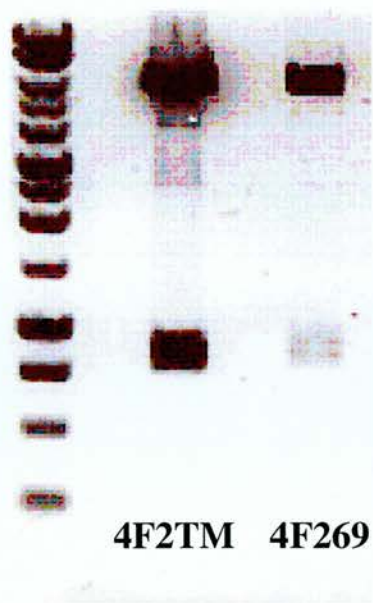


Figure 3.3.2. 69CYTO and 69TM subcloned from pcDNA 1 to pcDNA 3.1.

A) Mini-prep showing insert in 5 colonies in subclone 69CYTO. Insert size 1543 base pairs. Colony 7 was selected for maxi prep. B) Mini-prep showing insert in all 10 colonies in subclone 69TM. Insert size 1681 base pairs. Colony 1 was selected for maxi prep.

A)



B)

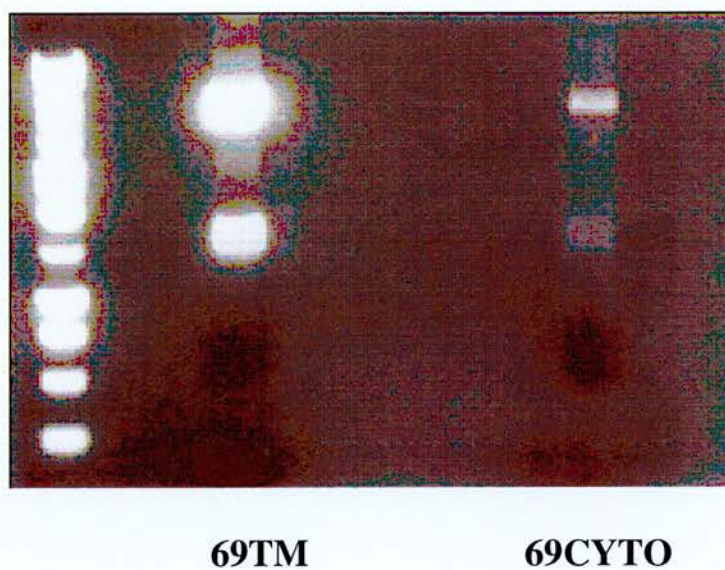


Figure 3.3.3 Maxi-preps of subclones

Maxi-preps were carried out on the subclones and the DNA was then digested using Bam H1 and Xho 1 restriction enzymes and the samples run on 1% agarose gels. The correct size inserts were present in all the subclones.

3.4 Production of stable cells expressing the CD98 chimeras

The plasmids verified by sequencing were transfected into subconfluent CHOK1 cells. Plasmid DNA(3 μ g) was transfected into these cells using lipofectamine plus in serum free media for 5 hours, as described in MATERIALS AND METHODS, section 2.5. For the subsequent 48 hours serum was added and transfectants selected in media with 1.2 mg/ml G418. Single clones selected from each construct were maintained in 1.2 mg/ml G418 and expanded. Clones were then screened by western blot analysis or by FACS analysis. The western blots were either probed with an anti-CD69 antibody or an anti-CD98 antibody depending on the extracellular domain expressed. The chimeras which required probing with the CD69 antibody were run on a non-reduced gel as the anti-CD69 antibody only recognizes the native protein. The resulting blots are shown in figure 3.4.1. For the FACS analysis, aliquots of cells were washed and resuspended in PBS containing either the 4F2 antibody (for CD98 and chimeras containing the extracellular portion of CD98) or anti-CD69 antibody (for CD69 and the chimeras containing the extracellular portion of CD69). Cells were incubated for 30 minutes at room temperature, washed and then incubated with species specific FITC conjugated secondary antibodies for 30 minutes at 4°C in the dark. After washing, the samples were finally suspended in PBS and analysed by flow cytometry. The resulting FAC'S analysis is shown in figure 3.4.2. The FACS and the western blotting confirmed that the CHOK1 stable cell lines were expressing each chimera at comparable levels. The clones showing equivalent wild type, mutated or truncated human CD98 expression by the FACS and western blotting analyses were then selected for use in this study.

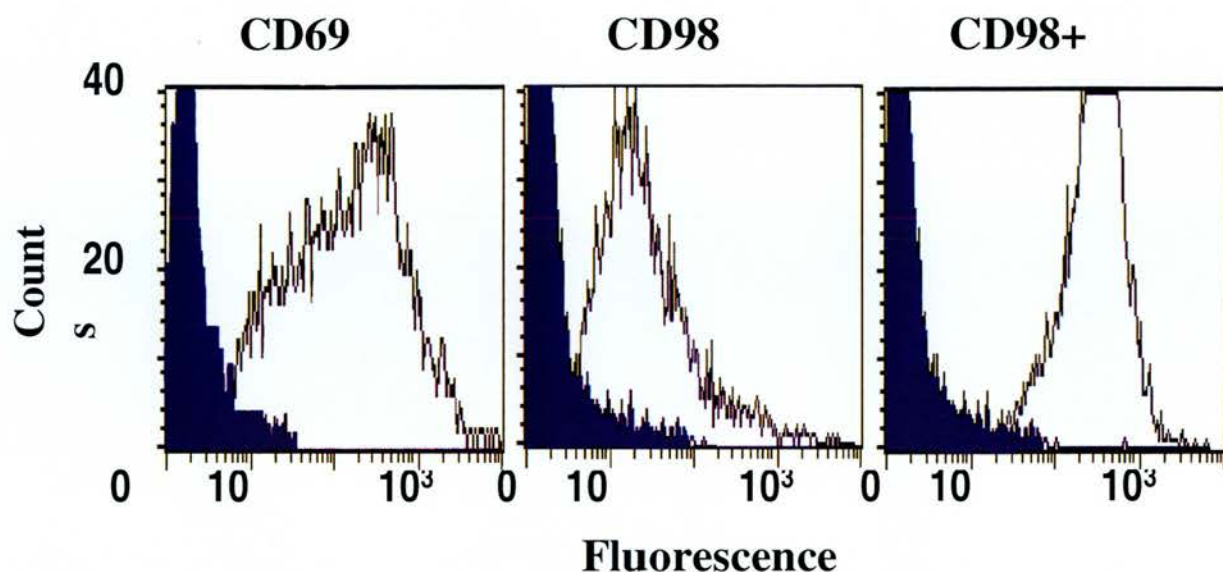


Figure 4.1.1a FACS analysis of CD69 or CD98 expression in stable CHOK1 clones.

CHOK1 cells were stably transfected with either CD69 or CD98. Expression of the constructs was verified by FACS analysis. Two clones stably expressing different cell surface levels of CD98 were selected by flow cytometry. CHOK1 cells stably expressing CD69, were used as a negative control. Cells were incubated with 4F2 antibody (for CD98) or an anti-CD69 antibody for 30 minutes at room temperature and then incubated with the appropriate FITC conjugated secondary antibody for 30 minutes at 4°C. Samples were analysed using a FACS Calibur™ (Becton Dickinson). Isotype controls IgG2a and IgG1 were used respectively to ensure there was no non-specific binding. Graphs are representative of three independent experiments.

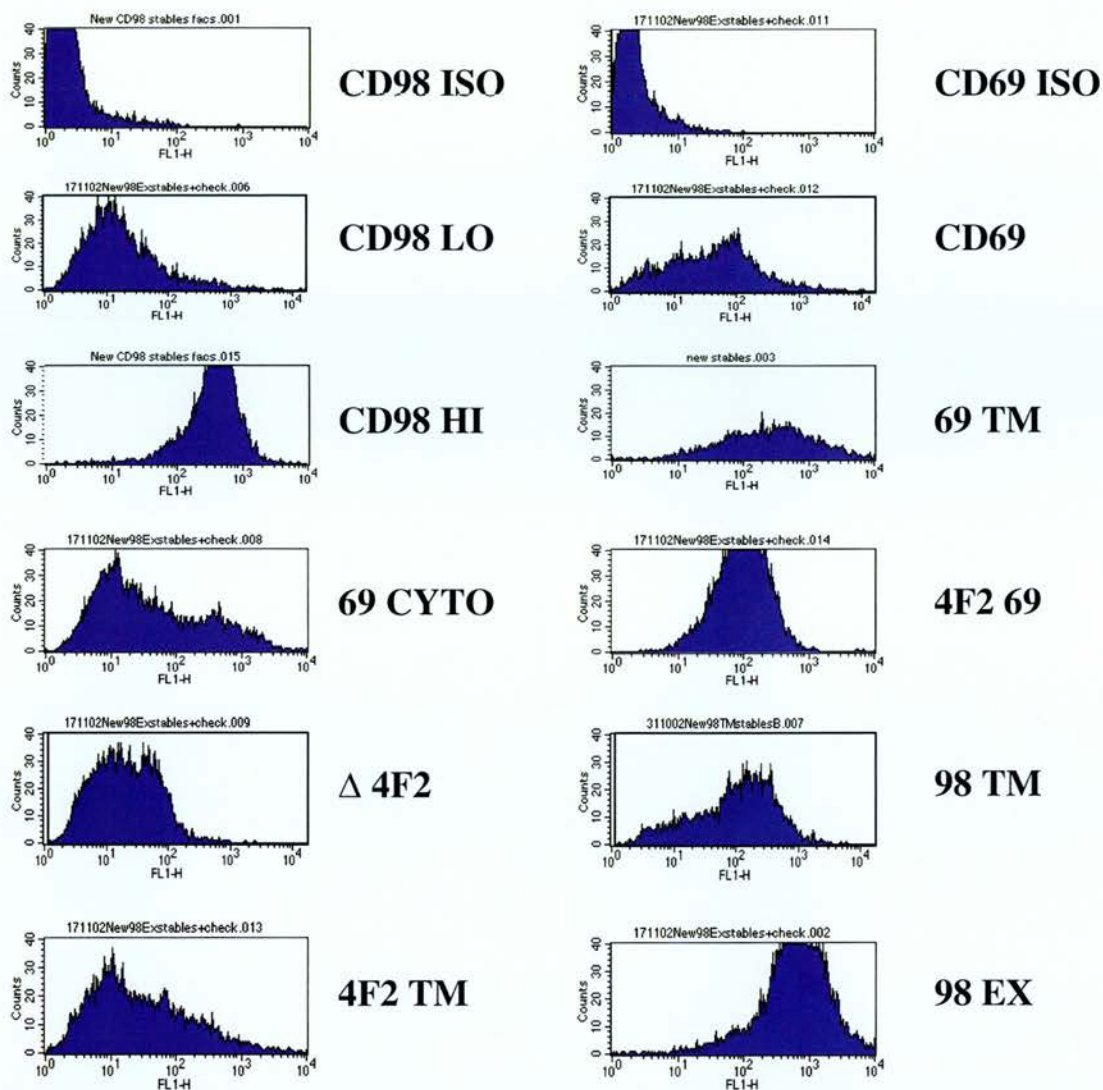


Figure 3.4.2 FACS analysis of the chimeric stable cell lines

Stably expressing CHOK1 cells were incubated with either anti-CD98 antibody or anti-CD69 antibody for 30 minutes at room temperature, washed and incubated with a corresponding species specific secondary FITC antibody for another 30 minutes at 4°C. The samples were analysed by FACS. These figures show that the cells were expressing each chimera at comparable levels.

CHAPTER 4

THE ONCOGENIC PROPERTIES OF CD98

4.0 INTRODUCTION

The survival and growth of a malignant tumour is a consequence of a phenomenon called “anchorage independence” (Stoker et al., 1968; Paul et al., 1978). This ability enables tumour cells to proliferate in the absence of adhesion to the extracellular matrix (ECM) proteins (Schwartz, 1997), and reflects the tendency of tumour cells to survive and grow in inappropriate locations *in vivo*, metastasise and invade. Incorrect localization is the characteristic that distinguishes malignant from benign tumours (Robbins et al., 1984).

Oncogenes are points on normal growth regulatory pathways that are constitutively activated by mutation or expression, (Schwartz, 1997). There is now increasing evidence that CD98 has oncogenic potential. The heavy chain of CD98 is expressed in most tissues and appears to be present in all tumour cells (Parmacek et al., 1989; Nakamura et al., 1999). NIH3T3 cells transfected with human CD98 were found to be capable of anchorage independent growth and CD98 transfected clones gave rise to tumours in athymic mice (Hara et al., 1999). Rintoul et al., (2002), have shown that cross-linking CD98 promoted anchorage independence. The ability of cancer cells to form colonies in semi-solid agarose is a fundamental feature of the transformed phenotype and anchorage independence. Colony formation shows good correlation with tumorigenesis and invasion (Carney et al., 1980). Full oncogenic transformation is believed to require both anchorage and serum independent growth, (Schwartz, 1997), facilitating signalling by integrin and growth factors.

The development of serum and anchorage dependence by CD98 was investigated using stably expressing CHOK1 cells suspended in semi-solid agarose medium.

The linkage between CD98 and integrins has been demonstrated in terms of cell membrane fusion. Ohgimoto et al, (1995) have shown via the comparison of amino-acid

sequence data that CD98HC and FRP-1 are the same molecule. Antibodies raised against FRP-1 enhanced cell fusion, induced by viruses such as HIV and Newcastle disease virus (Tabata et al., 1994). Okamoto et al., (1997) demonstrated that an anti CD98HC antibody (HBJ127) was able to inhibit fusion by the human parainfluenza type-2 virus, but also enhanced fusion induced by certain other viruses. This led to the conclusion that the heavy chain of CD98 was “multifunctional”.

An important feature of various oncogenes is their ability to activate signalling pathways downstream of integrins and integrin-activated tyrosine kinases. Phosphoinositol 3-OH kinase (PI3-Kinase) has been identified to have a critical role in compensating the cell survival and growth signals normally provided by the cell-matrix interaction (Wei et al., 2001). CD98 activity in haematopoietic cells could be inhibited by tyrosine kinase inhibitors suggesting that tyrosine kinase activation is involved in CD98 signalling (Warren et al., 1996). Rintoul et al, (2002), showed that when CD98 was cross-linked in SCLC cells using the monoclonal antibody 4F2, PI3-kinase activation was stimulated. PI3-kinase has also been shown to promote anchorage-independent growth acting through PKB (Moore et al., 1998).

PI3-Kinase activation and transformation was investigated using the CD98/CD69 chimeras in order to determine which part of the CD98 molecule is essential for these processes.

In cell anchorage-dependent survival and growth, the importance of the integrin/tyrosine kinase signalling pathway has been demonstrated by introducing constitutive active form of Focal adhesion kinase (FAK) into anchorage dependent cells and rendering them anchorage independent (Frisch et al., 1996). FAK is a tyrosine kinase that is rapidly activated following integrin-mediated attachment to the ECM and is thought to play an essential role in integrin signalling (Clark and Brugge, 1995; Schwartz et al., 1995).

Phosphorylation of FAK was investigated in terms of phenotypic changes and to find out which part of the CD98 molecule is crucial for the formation of focal adhesion complexes.

The importance of the cytoskeletal organization in transformed cells appears to be an issue which gives rise to conflicting views on this subject. Loss of anchorage independence and growth control is a common feature of cancer cells as mentioned above. Boyd et al., (1995) suggested that cytoskeletal components may play a role in the uncoupling cell shape and growth control. However, it has been extensively documented that an intact actin cytoskeleton is essential for signal transduction and integrin dependent focal adhesion (Bershadsky et al., 1996). In order to determine which hypothesis rings true for CD98, the actin cytoskeleton and another component of the cytoskeleton, the microtubular system were examined using CHOK1 cells stably transfected with either CD98 or CD69 and immunofluorescence staining.

4.1 Expression of CD98 induces anchorage independence and serum independent growth.

4.1.1 Clonal growth is dependent on level of CD98 expression.

CD98 is highly expressed on the surface of most tumour cells irrespective of tissue origin (Bellone et al., 1989; Dixon et al., 1990). The ability of cancer cells to proliferate in the absence of adhesion to extracellular matrix proteins is termed anchorage independent growth (Stoker et al., 1968, Paul et al., 1978 and Schwartz., 1997). Anchorage independence reflects the tendency of tumour cells to metastasise and grow in inappropriate locations *in vivo*. This property is the basis of the agar suspension technique used to clone transformed cells (Sanders and Burford, 1964, Stoker et al., 1968). The ability of cancer cells to form colonies in a semi solid agarose medium is a fundamental feature of the transformed phenotype.

Two clones stably expressing different cell surface levels of CD98 were selected by flow cytometry as shown in figure 4.1.1a. CHOK1 cells stably expressing CD69, another type II transmembrane protein was used as a negative control. Cells were incubated with 4F2 antibody (for CD98) or an anti-CD69 antibody for 30 minutes at room temperature and then incubated with the appropriate FITC conjugated secondary antibody for 30 minutes at 4°C. Samples were then analysed using a FACS Calibur™ (Becton Dickinson). Isotype controls IgG2a and IgG1 were used respectively to ensure there was no non-specific binding.

In order to investigate the role CD98 plays in cancer, the effect of over-expressing human CD98 heavy chain in CHOK1 cells on anchorage-independent growth in soft agar was examined as described in MATERIALS AND METHODS. Colonies of 4 or more cells were counted and cloning efficiency was calculated as described in MATERIALS AND METHODS. Figure 4.1.1b. shows representative pictures of colonies seen after 4 days in 1% FCS. They show the difference between cells overexpressing CD69 and CD98 and the difference a higher level of surface expression has on the size of colonies. Figure 4.1.1c shows low power pictures of the colonies

produced by overexpression of CD69, CD98 and CD98+ in CHOK1 cells and confirms the above observations. Figure 4.1.1d shows a time course of cloning efficiency, between CHOK1 cells expressing CD98 and CD69. The CHOK1 cells overexpressing CD98 showed cloning efficiencies higher than those seen in CHOK1 cells overexpressing CD69 regardless of the number of days suspended in 0.3 % agarose, (day 2, 27.20 ± 6.217 and 2.208 ± 1.597 respectively compared to day 4, 40.37 ± 3.573 and 2.0 ± 1.2 and day 6, 20.92 ± 4.698 and 0.68 ± 0.68). When these cells were left to grow for 10 days in culture, CHOK1 cells overexpressing CD98 showed a higher saturation density compared to CHOK1 cells overexpressing CD69 (Figure 4.1.1e). The cloning efficiency of CD98 expressing cells in 1% FCS was significantly higher than the control CD69 stably transfected CHOK1 cells (figure 4.1.1f). The level of CD98 expression also affected the cloning efficiency as clonal growth was greater in the clone with the highest level of CD98 surface cell expression. For all subsequent experiments the high expressing CD98 clone was used unless otherwise indicated.

These findings indicate that the CD98 heavy chain promotes anchorage independence and this property can be affected by the level of CD98 cell surface expression.

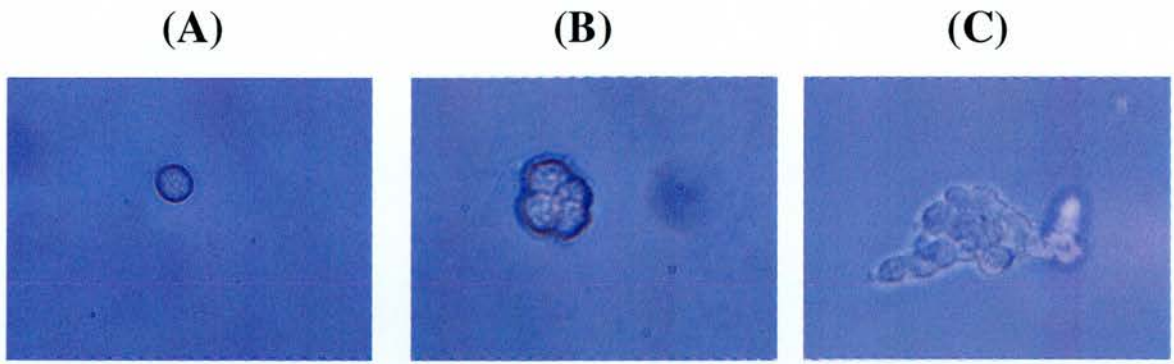


Figure 4.1.1b Colonies visible after 4 days.

CD98 or CD69 were stably transfected into CHOK1 cells which were then suspended in 0.3% agarose over a layer of 0.5% agarose in DMEM containing 1 %FCS, as described in MATERIALS AND METHODS. (A) CD69 stables, (B) CD98 stables and (C) CD98+ stables. Images were taken at 32 x magnification with a phase microscope.

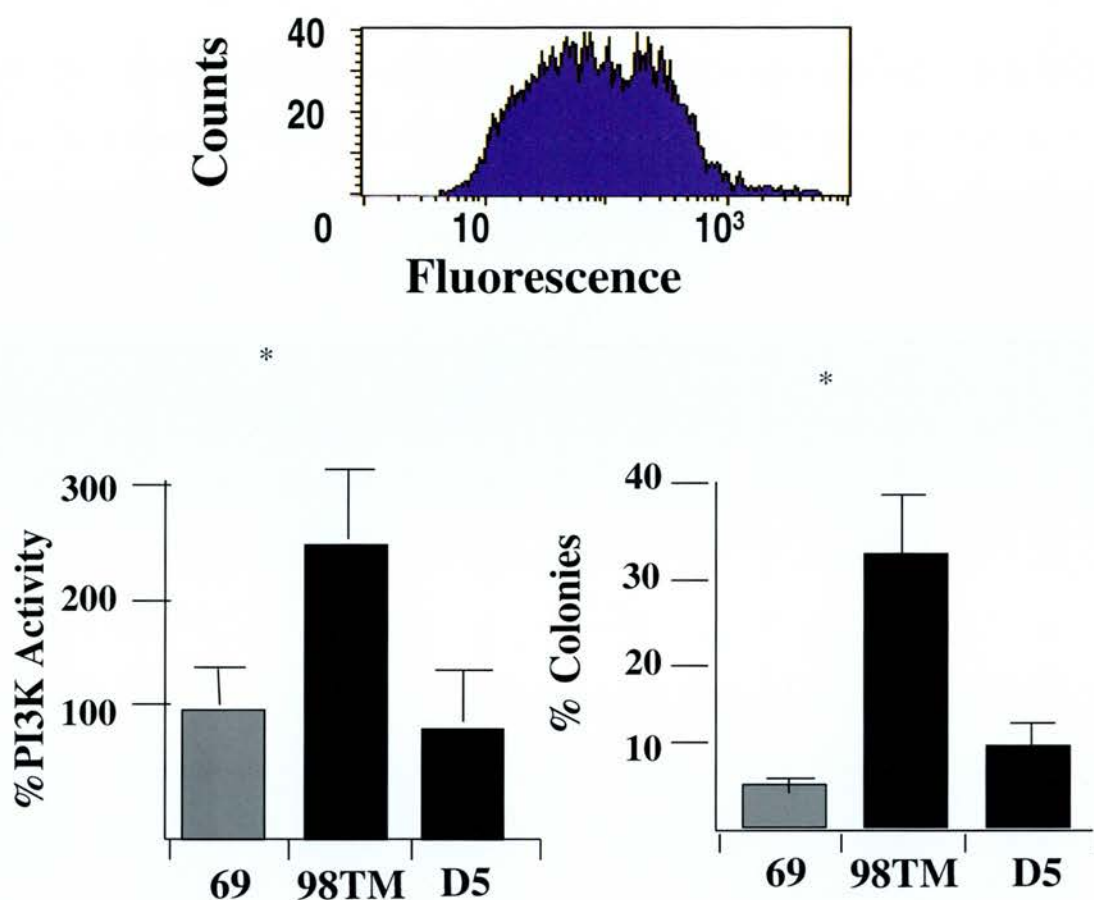


Figure 4.2.3. Amino acids 82-26 of CD98 are critical for PI3Kinase activity and anchorage independent growth.

CHOK1 cells stably transfected with D5 were analysed to determine whether these cells could promote PI3Kinase activity and transformation.

A) Expression of D5 was determined by flow cytometric analysis as described in MATERIALS AND METHODS using the monoclonal antibody 4F2.

B) The effect of stable D5 expression on PI3-kinase activity and anchorage independent growth was compared with 98TM and CD69 using the method described in MATERIALS AND METHODS. *= $P < 0.05$ (ANOVA). These graphs represent the mean \pm S.E.M. of four independent experiments.

CD69

CD98

CD98+

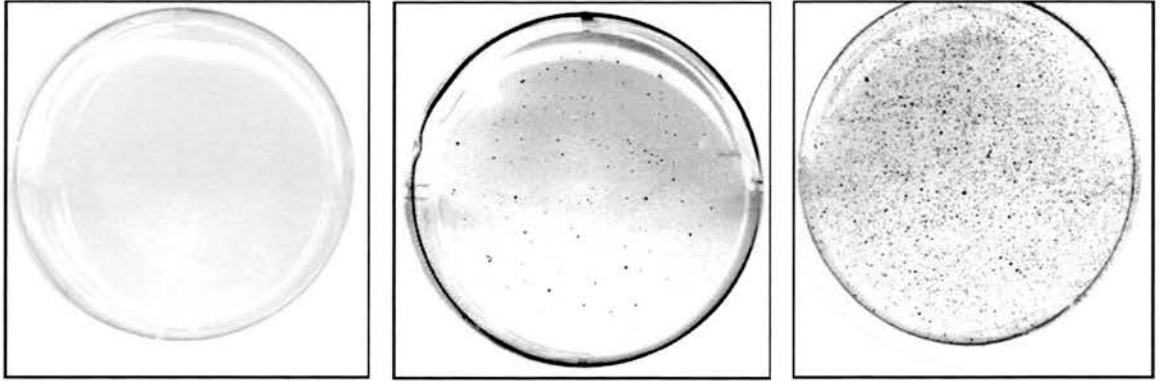


Figure 4.1.1c CD98 increase clonal growth in CHOK1 cells.

CD98 or CD69 was stably transfected into CHOK1 cells and then suspended in 0.3% agarose over a layer of 0.5% agarose in DMEM containing 1% FCS, described in MATERIALS AND METHODS. After 4 days, colonies of cells were stained using 5mg/mlMTT for 30 minutes and then visualised by using a scanner.

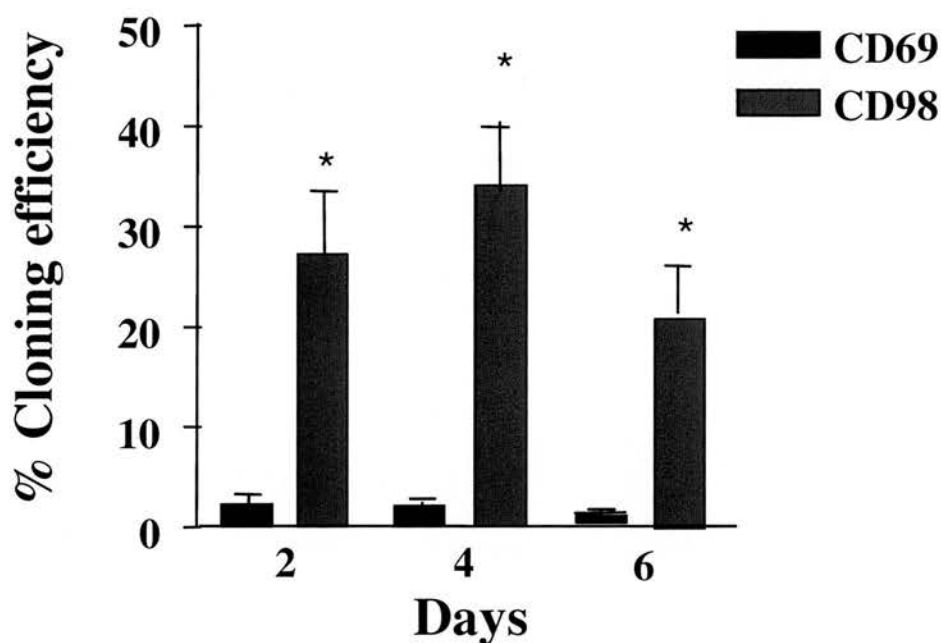


Figure 4.1.1d Time course of clonal growth of CHOK1 cells overexpressing CD98 and CD69.

CD98 or CD69 was stably transfected into CHOK1 cells and then suspended in 0.3% agarose over a layer of 0.5% in DMEM containing 1% FCS, described in MATERIALS AND METHODS. By counting the number of 4 cell colonies present on days 2, 4 and 6 the cloning efficiency was then calculated as described in MATERIALS AND METHODS. Results represent the mean \pm SEM of four independent experiments.

* = $P < 0.05$

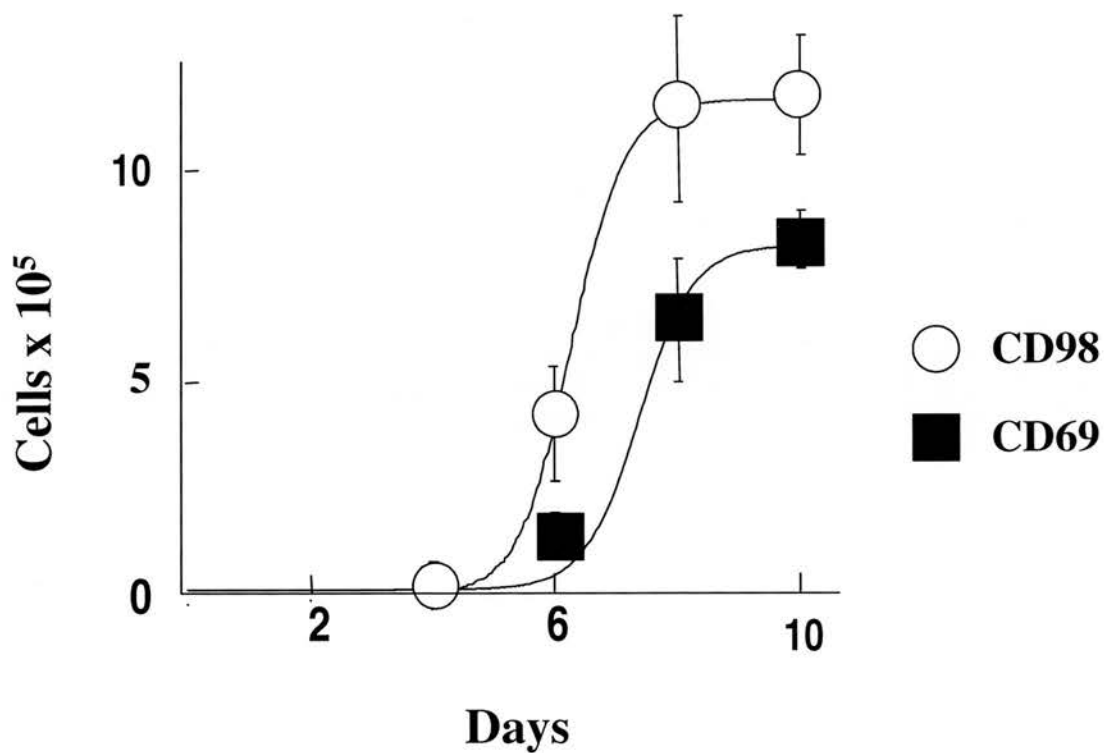


Figure 4.1.1e Liquid growth of CHOK1 cells overexpressing CD98 or CD69.

CHOK1 cells overexpressing CD98 or CD69 were left to grow for 10 days in complete media. On days 2, 4, 6 8 and 10, the cells were harvested and counted using a Coulter counter (Coulter Electronics, Luton, UK.)

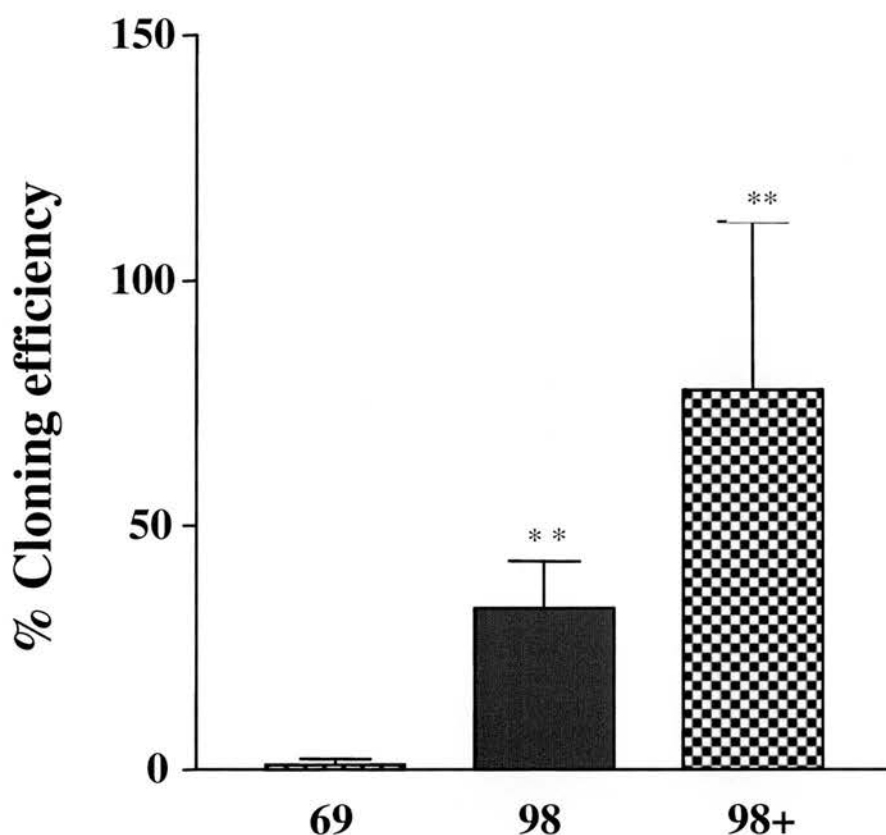


Figure 4.1.1f CD98 increases clonal growth in CHOK1 cells.

CD98 or CD69 was stably transfected into CHOK1 cells and then suspended in 0.3% agarose over a layer of 0.5% agarose in DMEM containing 1% FCS, as described in MATERIALS AND METHODS. On day 4 cloning efficiency was established by counting the number of colonies with four cells or more and carrying out the calculation for cloning efficiency, described in MATERIALS AND METHODS. Results represent the mean \pm SEM of four independent experiments. * *= clonal growth is significantly different in CHOK1 cells overexpressing CD98 compared to CD69 and CD98+, ($P < 0.01$).

4.1.2 Overexpression of the CD98 heavy chain results in serum independent clonal growth.

Oncogenes commonly by-pass the requirements for serum independence as well as anchorage independence and this phenomenon was examined, (figure 4.1.2). CHOK1 cells stably expressing the CD98HC were suspended in 0.3% agarose over 0.5% agarose in DMEM containing either 1% or 0% FCS. After 6 days, colonies greater than 4 cells were counted using light microscopy. We found that even in serum free conditions CD98 heavy chain could still support anchorage independent growth.

The above results show that over-expression of CD98 results in serum independent and anchorage independent clonal growth.

4.1.3 CD98 stimulated anchorage independent growth is PI3-kinase dependent.

Moore et al., (1998) found that PI3-kinase acting through PKB could promote anchorage independent growth and having determined that the CD98HC could promote clonal growth, I decided to examine the effect of PI3-kinase inhibition on CD98 stimulated anchorage independent growth. Colony assays were carried out in the presence of the PI3-Kinase inhibitor LY294002. By inhibiting PI3-Kinase activation using LY294002, there was a marked concentration dependent inhibition of colony formation in semi-solid agarose medium of CHOK1 cells stably over expressing CD98 in 1% FCS. ($IC_{50} = 2.1\mu M$, Figure 4.1.3).

The above results show that over-expression of CD98 promotes anchorage independent growth which is sensitive to the PI3-kinase inhibitor LY294002.

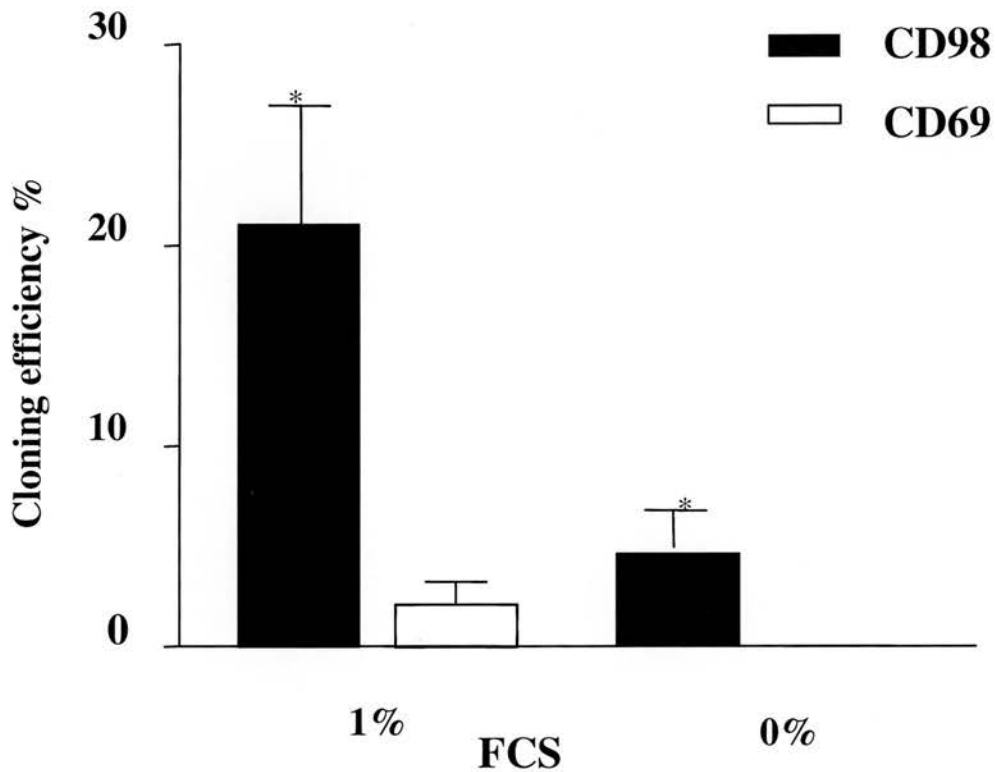


Figure 4.1.2. CD98 stimulated clonal growth is serum independent.

CHOK1 cells stably transfected with CD98 (filled bars) or CD69 (open bars) were suspended in 0.3% agarose over a layer of 0.5% agarose in DMEM containing FCS as indicated as described in MATERIALS AND METHODS. After 6 days colonies greater than 4 cells were counted using a light microscope. Results are expressed as % cloning efficiency and are the mean \pm SEM of 3 independent experiments. * = $P < 0.05$ (student t-test). CD98 transfected cells cloning efficiency is significantly different to that of CD69 transfected cells.

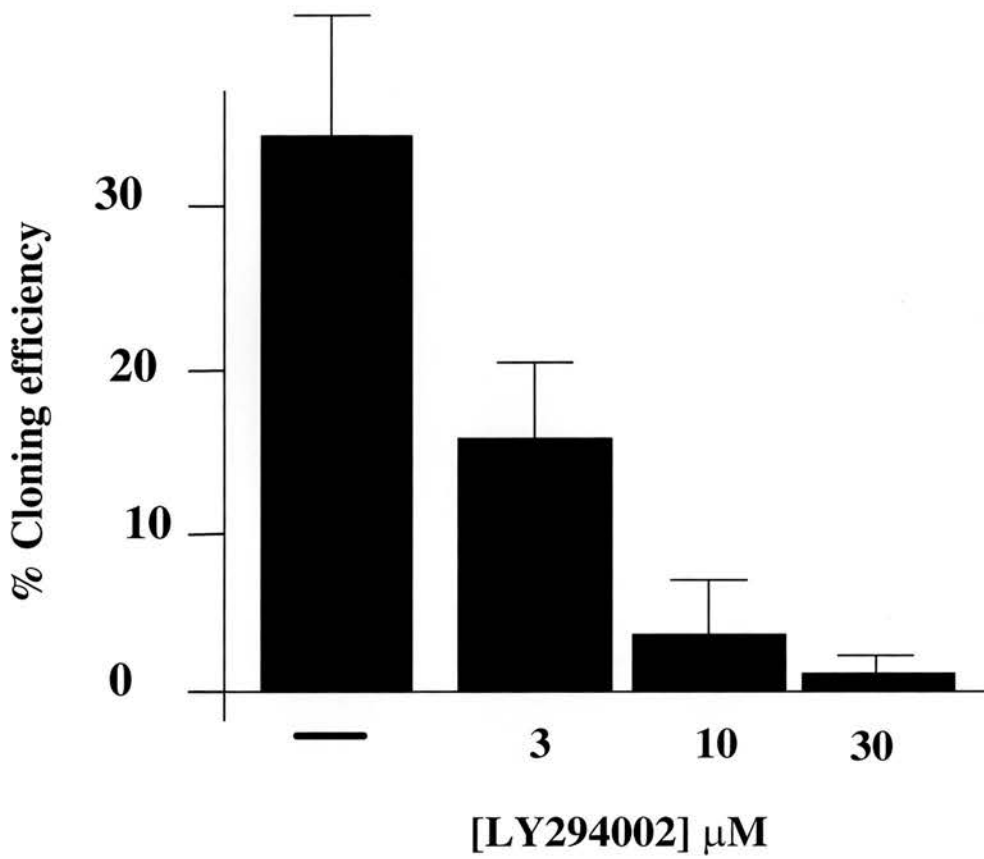


Figure 4.1.3 Effect of LY294002 on CD98 stimulated clonal growth.

The clonal growth of CHOK1 cells after 4 days expressing CD98 in semi-solid 0.3% agarose medium containing 1% FCS was determined at different concentrations of LY294002 as indicated. Results are expressed as % cloning efficiency and are the mean \pm SEM of 3 independent experiments.

4.1.4 The CD98 heavy chain stimulates PI3-kinase activation.

PI3-kinase plays a key role in integrin activation, cellular activation and transformation (Carpenter and Cantley, 1996). Rintoul et al., (2002) found that by cross-linking CD98 using the monoclonal antibody 4F2, PI3-kinase and its product PI (3,4,5) P₃ was stimulated in SCLC cells. In order to examine this interaction further, the effect of over-expressing CD98 in CHOK1 cells on PI3-kinase activity was investigated. CD98 or CD69 stably expressing CHOK1 cells were lysed and PI3-kinase was immunoprecipitated using a p85 α monoclonal antibody, (figure 4.1.4(B)). Activity was measured using an *in vitro* kinase assay and lipids were resolved using thin layer chromatography as described in MATERIALS AND METHODS. Expression of CD98 significantly increased PI3-kinase activity by 2-2.5 fold compared to CD69 and wild type CHOK1 cells (figure 4.1.4 (A)). CD98 expression also increased phosphorylation of PKB, the downstream effector of PI3-kinase, (figure 4.1.4 (D)).

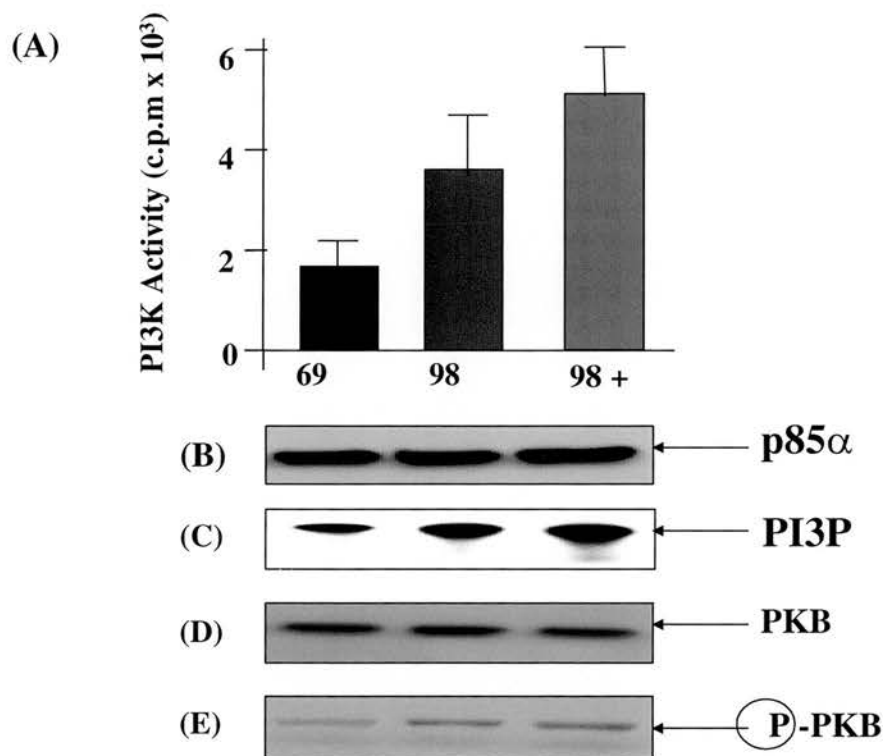


Figure 4.1.4 CD98 activates PI3-Kinase.

(A) Quiesced CHOK1 cells stably expressing CD69 or CD98 (low and high (+) expressing cells) were lysed, protein equilibrated and PI3-kinase activity was measured from p85α immunoprecipitates using an *in vitro* kinase assay as described in MATERIALS AND METHODS. Radiolabelled PI3P was resolved by thin layer chromatography, visualised by autoradiography and ^{32}P PI3P scraped from the plate and quantified by liquid scintillation counting. Results are expressed as cpm and represent the mean \pm SEM of four independent experiments. (B) Aliquots of lysate were run on SDS-PAGE and western blotted with p85α, to ensure equal immunoprecipitation. (C) An autoradiograph showing the 3-phosphorylated product [PI(3)P]. Lysates were western blotted with anti-PKB antibody (D) or (E) anti-pS473PKB.

4.2 Investigating structure/function relationship with CD98/CD69 chimeras

4.2.1 The transmembrane domain (amino acids 82-104) of CD98 heavy chain is necessary and sufficient for PI3-kinase activation and the elevation of intracellular PI (3, 4, 5)P₃ formation.

The CD98/CD69 chimeras, kindly donated by C.Fenzik (as described in chapter 1) were used to investigate the structure/function relationship of CD98 and PI3-kinase activation. CHOK1 cells stably expressing CD98/CD69 chimeras were quiesced in serum free medium for twelve hours prior to lysis and PI3-kinase activity was measured by an *in vitro* kinase assay as described in MATERIALS AND METHODS. PI(3,4,5)P₃ production was also measured using a radioisotope dilution assay as described previously (van der Kaay et al.,1999). This assay is based on radioligand displacement of radiolabelled Ins(1,3,4,5)P₄ from binding sites present in platelets that display high affinity and specificity for Ins(1,3,4,5)P₄. Following a Folch extraction of cellular phospholipids, Ins(1,3,4,5)P₄, the polar head group of PtdIns (3,4,5)P₃, is released by alkaline cleavage and its mass can be measured by radioligand displacement using a calibration curve obtained using unlabelled Ins(1,3,4,5)P₄ standards. Figure 4.2.1a. shows again that expression of CD98 increases PI3Kinase activity by 2-2.5 fold compared to wild type CHOK1 cells and CHOK1 cells overexpressing CD69. It also shows that the transmembrane domain of CD98 is sufficient and necessary to activate PI3-kinase. Figure 4.2.1b demonstrates that again the transmembrane domain of CD98 is sufficient to promote PI(3,4,5)P₃ production. This became apparent because the chimera 98TM (which has the transmembrane domain of CD98 and the extracellular and cytoplasmic domains of CD69) and the truncated mutant Δ 4F2 (which has the cytoplasmic domain amino acid 2-77 deleted) were both able to stimulate PI3-kinase activation and PI(3,4,5)P₃ production. These chimeras, 4F2TM, Δ 4F2 and 98TM also showed a 2-2.5 fold increase in PI3Kinase activity and PIP₃ production compared to

wild type CHOK1 cells and CHOK1 cells overexpressing CD69. However the chimera 69TM in which the transmembrane domain of CD98 is substituted with the transmembrane domain of CD69 could not activate PI3-kinase or the generation of its product.

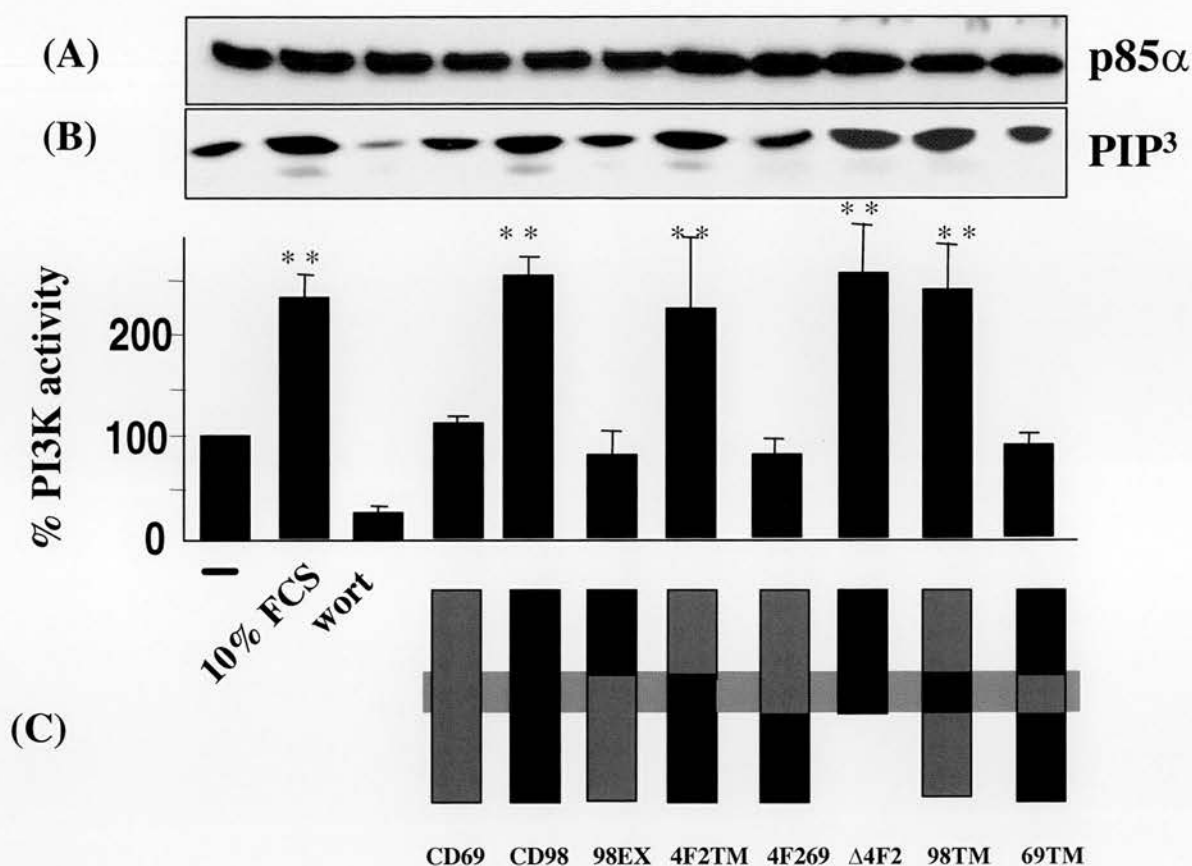


Figure 4.2.1a The transmembrane domain of CD98 heavy chain (amino acids 82-104) is necessary and sufficient for PI3K activation.

Quiesced CHOK1 cells stably expressing CD69, CD98 or CD98 chimeras were lysed, and PI3-kinase activity was measured from p85a immunoprecipitates using an *in vitro* kinase assay as described in MATERIALS AND METHODS. (A) Aliquots of lysate were run on SDS-PAGE and western blotted with p85a, to ensure equal immunoprecipitation. Radiolabelled PIP³ was resolved by thin layer chromatography, visualised by autoradiography and ³²P PIP³ scraped from the plate and quantified by liquid scintillation counting. (B) An autoradiograph showing the 3-phosphorylated product PIP³. (C) Results are expressed as % activity of control untransfected cells and represent the mean \pm SEM of four independent experiments. The first bar represents

wild type CHOK1 cells. Wild type CHOK1 cells were also treated with 100nm wortmannin or 10% FCS for 10 mins at 37°C, as negative and positive controls respectively, bars two and three. ** = PI3-Kinase activity is significantly different to wild type CHOK1 cells ($P < 0.01$ ANOVA).

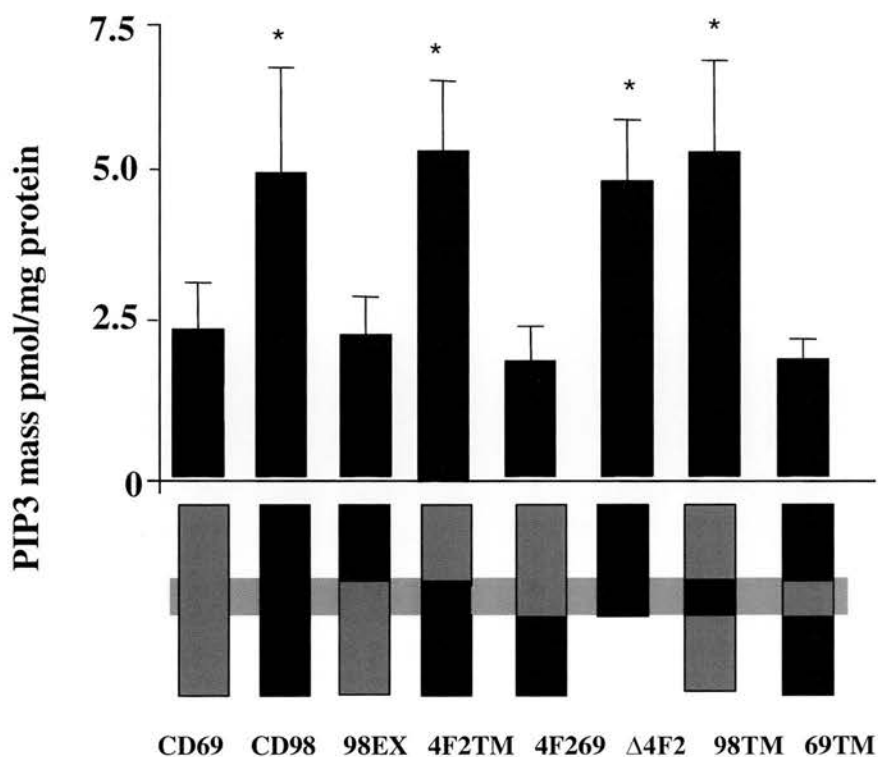


Figure 4.2.1b The transmembrane domain (amino acids 82-104) of CD98 heavy chain is sufficient and necessary for intracellular PI(3,4,5)P3 production.

The effect of chimeras on intracellular PI(3,4,5)P3 levels. PI(3,4,5)P3 levels were measured in quiesced CHOK1 cells stably expressing CD69, CD98 or CD98 chimeras. The level of PI (3, 4, 5) P3 was quantified by an isotope dilution assay as described in MATERIALS AND METHODS. Results are expressed as pmol/mg protein and represent the mean \pm SEM of four independent experiments. * = PIP3 production is significantly different to CHOK1 cells overexpressing CD69. ($P < 0.05$ ANOVA).

4.2.2 The transmembrane domain (amino acids 82-104) of the CD98 heavy chain is necessary and sufficient for colony formation.

The structure/function relationship of CD98 in the phenomenon of anchorage independence was examined using the CD98/CD69 chimeras. CHOK1 cells stably transfected with the CD98/CD69 chimeras were suspended in 0.3% agarose layered over a layer of 0.5% agarose in 1% FCS and were counted after 6 days using a light microscope as described in MATERIALS AND METHODS. All the chimera clones containing the transmembrane domain of CD98 showed cloning efficiencies comparable with CHOK1 cells over-expressing wild type CD98 whereas the chimeras containing the transmembrane domain of CD69 had cloning efficiencies which were not significantly different from untransfected CHOK1 cells or the CD69 stables, (figure 4.2.2). The chimeras Δ 4F2 (mutant with the cytoplasmic domain deleted) and 98TM (containing the CD98 transmembrane domain and the extracellular and cytoplasmic domains of CD69) both markedly enhanced colony formation when stably expressed in CHOK1 cells (30.2 ± 6.2 and 33.2 ± 3.4 respectively compared to control 2.2 ± 1.7 , mean of four independent experiment done in triplicate mean \pm S.E.M.). However the opposite equivalent chimera 69TM (in which the transmembrane domain within CD98 is replaced with the transmembrane domain of CD69) did not enhance colony formation (9.6 ± 6.2 n = 4 in triplicate mean \pm S.E.M.). The difference between CD98 clones and wild type CHOK1 cells and CD69, 69TM and 98EX clones is significant as measured by one way ANOVA with a P value ranging from <0.05 - 0.001 .

Therefore the transmembrane domain of the CD98 heavy chain is sufficient and necessary to stimulate PI3-kinase activity, increase intracellular PIP3 levels and promote anchorage independent growth.

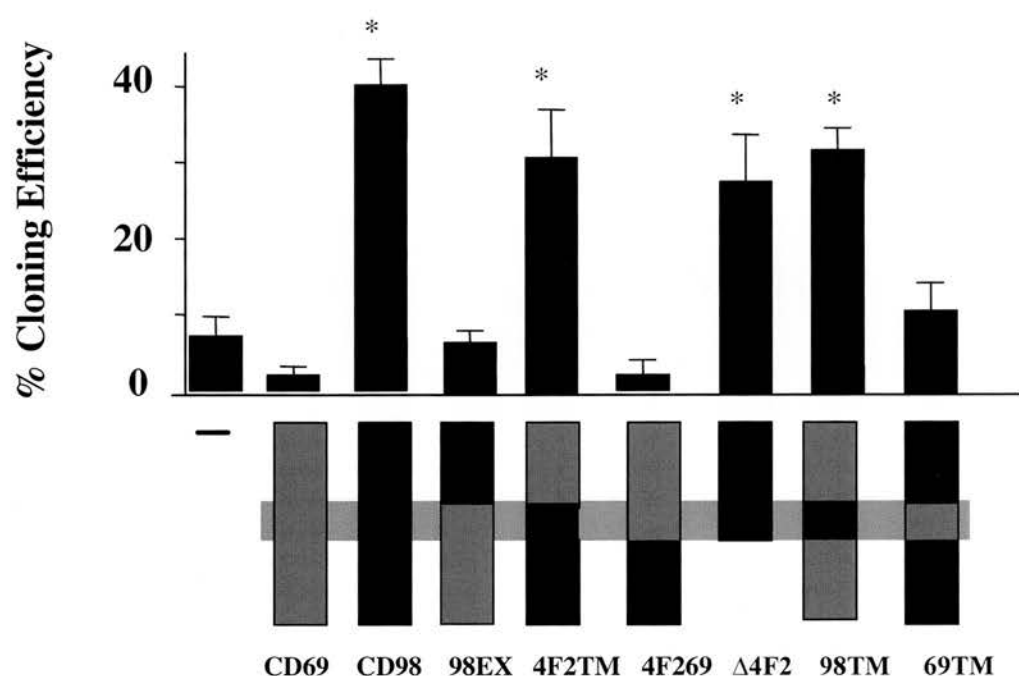


Figure 4.2.2 The transmembrane domain (amino acids 82-104) of CD98 heavy chain is necessary and sufficient to promote anchorage independent growth.

CHOK1 cells stably expressing CD69, CD98 or CD98 chimeras were grown in colonies for 6 days in 0.3% agarose over a layer of 0.5% agarose in 1% FCS culture medium as described in MATERIALS AND METHODS. Colonies greater than 4 cells were counted by light microscopy. Results are expressed as % cloning efficiency and are the mean \pm SEM of four independent experiments.* = Cloning efficiency is significantly different from wild type CHOK1 cells (-). ($P = < 0.05$ ANOVA).

4.2.3 Amino acids 82-86 of CD98 are critical for PI3-Kinase activation and anchorage independent growth.

The above finding was then examined further using the CD98 truncation mutant D5. The D5 mutant lacks amino acids 1-86 but is well expressed in CHO cells, as detected by flow cytometry. While the chimera 98TM (which has the extracellular and cytoplasmic domains of CD69 and the transmembrane domain of CD98) and the Δ 4F2 mutant (which lacks the putative cytoplasmic tail (amino acids 2-77)), were able to stimulate PI3-Kinase activation and promote anchorage independent growth in semi-solid agarose medium (Figure 4.2.1a/b and 4.2.2), the D5 mutant in contrast failed to stimulate PI3-kinase activity when stably expressed in CHO K1 cells and did not support anchorage independent growth (Figure 4.2.3).

The above results suggest that the five amino acids 82-86 at the putative cytoplasmic tail/transmembrane domain interface are required for PI3-Kinase activation and transformation.

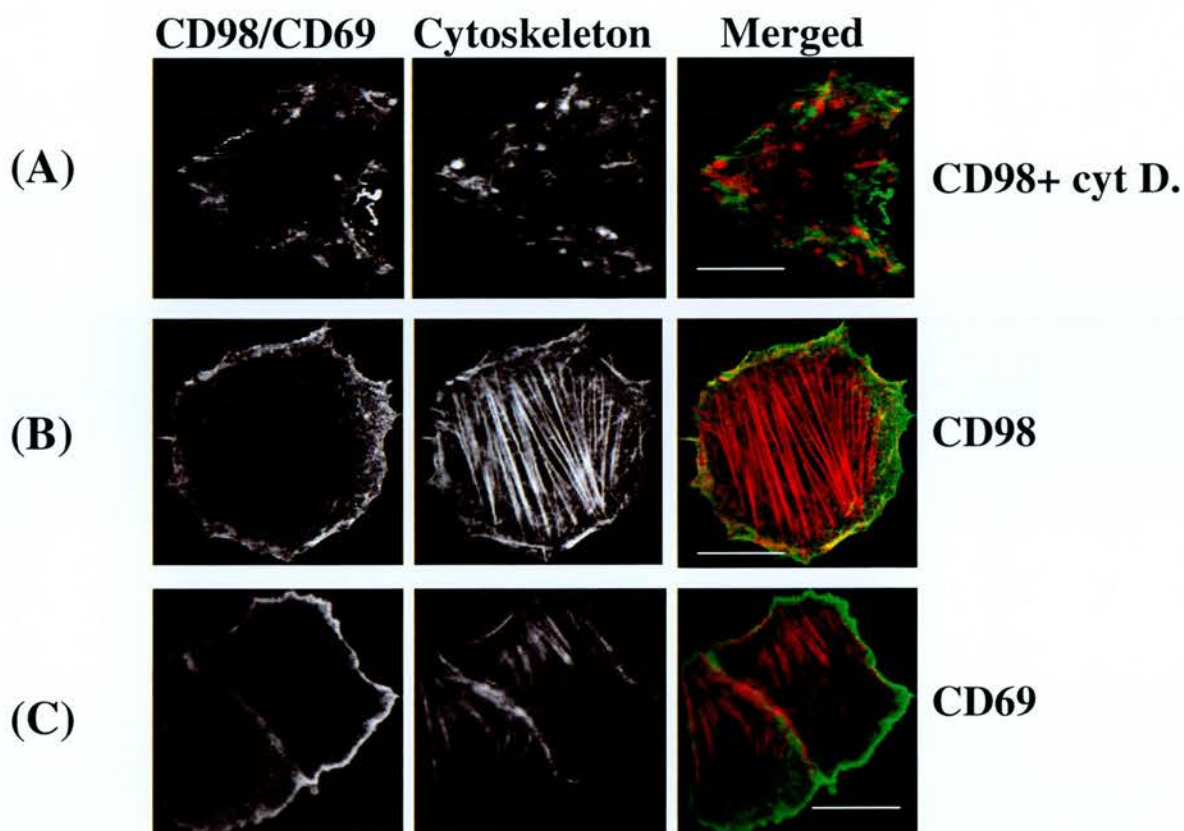


Figure 4.3.1 CD98 has no effect on the cytoskeleton of CHOK1 cells.

CHOK1 cells stably transfected with the CD98 or CD69 were fixed and incubated with rhodamine phalloidin and CD98 or CD69 antibodies and secondary alexa fluor antibodies as described in MATERIALS and METHODS. White bars, 28.6 μm . Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica).

(A) Disruption of the cytoskeleton by cytochalasin D treatment. CD98 expressing cells treated with 2 μM cytochalasin D for 30mins labelled with (4F2-green) and rhodamine phalloidin (red). B) Effect of CD98 on the cytoskeleton. CD98 cells labelled with (4F2-green) and the cytoskeleton (red). (C) Effect of CD69 on the cytoskeleton. CD69 expressing cells labelled with (FN50-green) and rhodamine phalloidin (red).

4.3 CD98 has no effect on the architecture of the cell.

There appears to be conflicting views on the importance of cytoskeletal organization in neoplastic transformed cells. A common change in transformed cells is the loss of anchorage dependence to the ECM or shape dependent controls on growth, (Benecke et al.,(1978). It has been hypothesised that cytoskeletal components may play a role in the uncoupling of cell shape and growth control (Boyd et al.,1995). However more recently it has been extensively documented that an intact actin cytoskeleton is essential for integrin dependent focal adhesion assembly and signal transduction (Bershadsky et al.,1996). Oncogenes appear to have the ability to bypass the need for anchorage independence and growth factors but can still activate integrin-dependent signalling. Therefore these hypotheses were examined in terms of whether over-expressing CD98 disrupted the actin cytoskeleton.

4.3.1 CD98 has no effect on the cytoskeleton of CHOK1 cells.

Quiesced CHOK1 cells stably transfected with CD98 or CD69 were incubated with a drug, which directly binds to actin, (rhodamine phalloidin) and the antibodies CD98 (4F2) or CD69 (FN50-FITC), described in MATERIALS AND METHODS and analysed by confocal microscopy (Figure 4.3.1) The actin cytoskeleton of CD98 expressing cells was chemically disrupted using 2µg/ml of Cytochalasin D for 30 minutes and visualised by rhodamine phalloidin. Cytochalasin D treatment resulted in the disruption of the filamentous appearance of actin within the cell with no radial microfilaments observed. This was then used as a control to compare the actin cytoskeleton of both CD98 and CD69 stably expressing CHOK1 cells. CD69 and CD98 expressing cells displayed a well organised actin assembly, with no obvious differences between the two cells types. Equally the microfilaments appeared to still be intact and thus there was no apparent disruption of the cytoskeleton.

In many cell types the actin cytoskeleton functions together with other cytoskeletal components, especially the microtubular system to induce signal transduction pathways and the assembly of focal adhesions. Bershadsky et al., (1996) found that in serum starved Swiss 3T3 cells; the microtubular system was well developed and had the typical radial organization but poorly organized actin bundles. However when the microtubular system was chemically disrupted using nocodazole, the assembly of focal adhesion and actin microfilament bundles was induced. These observations lead to the examination of whether the CD98 stables (which have an intact actin cytoskeleton) have a disrupted microtubule network.

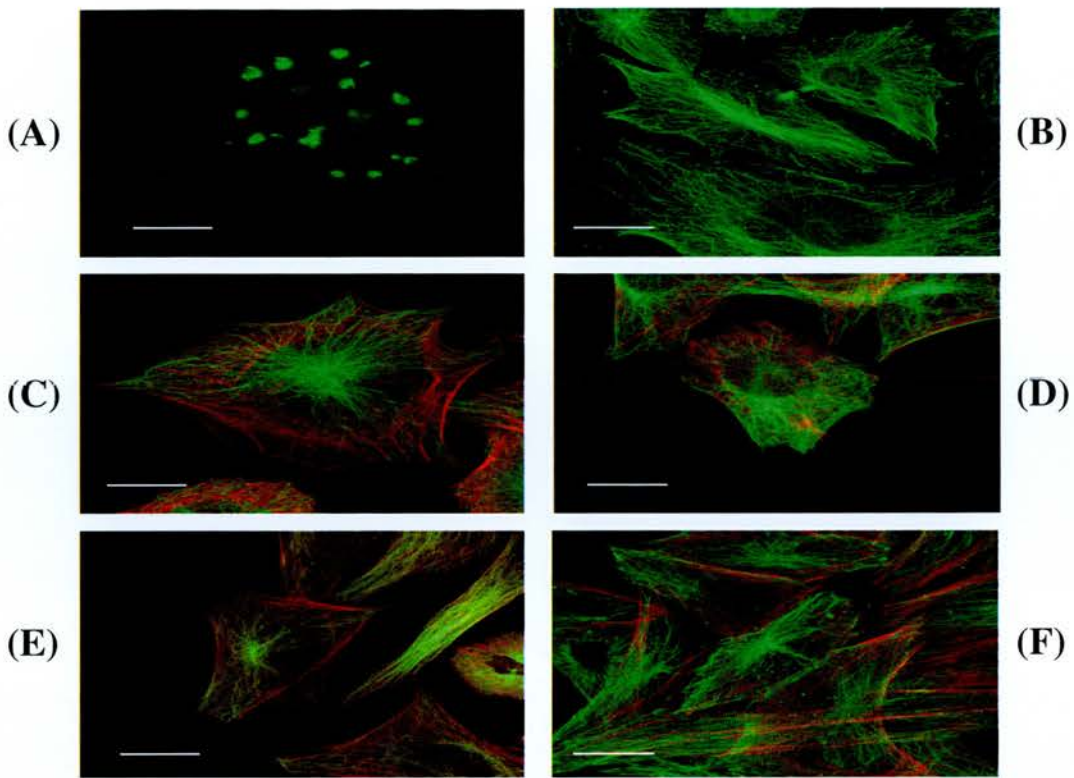


Figure 4. 3. 2 .CD98 has no effect on the microtubule network.

CHOK1 cells were stably transfected with CD98 or CD69 or the chimeras and wild type CHOK1 cells were labelled with α tubulin or rhodamine phalloidin. White bars 28.3 μm. Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica).

(A) Wild type CHOK1 cells were treated with 10 μM nocodazole for 30 minutes and then incubated with tubulin antibody (green).

(B) Wild type CHOK1 cells were incubated with tubulin antibody (green).

(C) CD98, (D) CD69, (E) 98TM stables and (F) wild type CHOK1 cells were incubated with antibodies against tubulin (green) and rhodamine phalloidin.

4.3.2 CD98 has no effect on the microtubular system of CHOK1 cells.

CHOK1 cells stably transfected with either CD98 or CD69 the CD98/CD69 chimeras and the wild type CHO K1 cells were fixed and labelled with rhodamine phalloidin and/or tubulin antibody described in MATERIALS AND METHODS and visualised by confocal microscopy. The microtubule network of wild type CHOK1 cells was disrupted using the microtubule disrupting drug, Nocodazole (10 μ M) for 30 mins. and then incubated with the tubulin antibody. This caused the characteristic tubulin paracrystals as shown in Figure 4.3.2 (A). This again was used as a control in comparison with CHOK1 cells stably expressing CD98, CD69 and the chimera 98TM (has the transmembrane domain of CD98 and the extracellular and cytoplasmic domain of CD69). There was no significant difference between the CD98, CD69, 98TM stables and the wild type CHOK1 cells and again the microtubule network of these cells was intact.

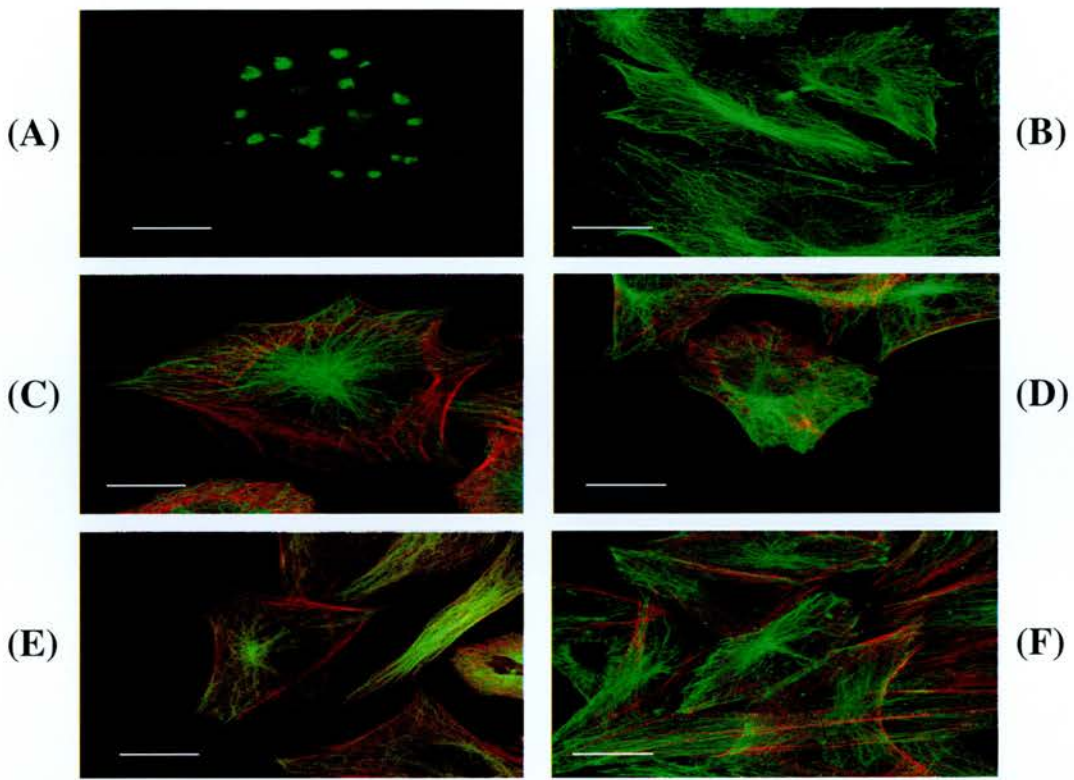


Figure 4. 3. 2 .CD98 has no effect on the microtubule network.

CHOK1 cells were stably transfected with CD98 or CD69 or the chimeras and wild type CHOK1 cells were labelled with α tubulin or rhodamine phalloidin. White bars 28.3 μ m. Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica).

(A) Wild type CHOK1 cells were treated with 10 μ M nocodazole for 30 minutes and then incubated with tubulin antibody (green).

(B) Wild type CHOK1 cells were incubated with tubulin antibody (green).

(C) CD98, (D) CD69, (E) 98TM stables and (F) wild type CHOK1 cells were incubated with antibodies against tubulin (green) and rhodamine phalloidin.

4.3.3 The transmembrane domain of the CD98 heavy chain is sufficient to induce phenotypic changes.

One of the earliest events in integrin mediated signal transduction is an increase in tyrosine phosphorylation of multiple cytoskeletal proteins and the enzymatic activation of the focal adhesion kinase (FAK) (Meng and Lowell, 1998). Therefore the effect of expressing CD98 in CHOK1 cells on the localisation of focal adhesion complexes was examined using confocal microscopy. CHOK1 cells stably expressing CD98 or CD69 or the chimera 98TM were incubated with rhodamine phalloidin and a P-FAK (Tyrosine 397) antibody as described in MATERIALS AND METHODS. There was a striking difference between CD98 stables and CD69 in terms of P-FAK localisation and the size of the focal adhesions (Figure 4.3.3). The CD98 heavy chain promoted larger and more extensive focal adhesion complexes in comparison to CD69. The transmembrane domain of CD98 also appeared to be sufficient to promote the changes seen with CD98 as the chimera 98TM also showed the same phenotype.

The above results have shown that CD98 transmembrane domain is sufficient to promote the formation of extensive focal adhesion complexes but does not appear to have any effect on the actin cytoskeleton or the microtubule network.

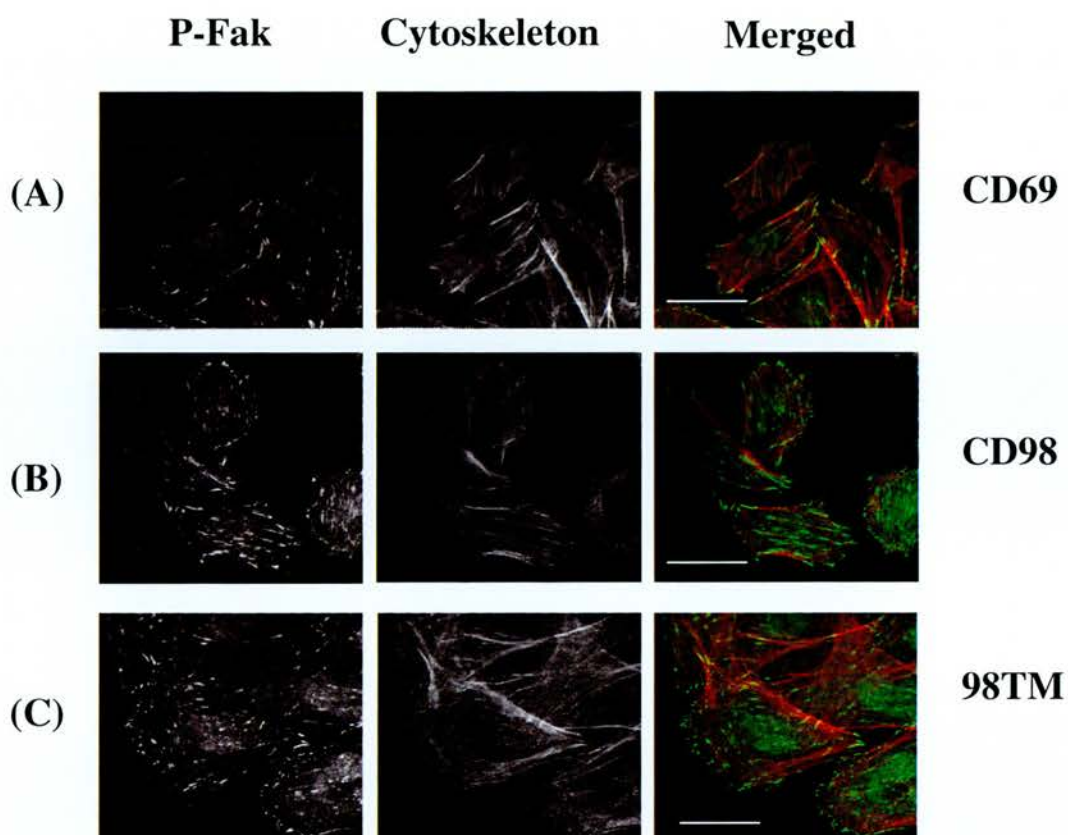


Figure 4.3.3 The transmembrane domain of CD98 is sufficient to induce phenotypic changes such as the promotion of large and extensive focal adhesion complexes

CHOK1 cells stably transfected with the CD98 or CD69 or 98TM were incubated with rhodamine phalloidin and P-Fak antibody, described in MATERIALS and METHODS. White bars, 28.6 μm . Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica).

(A) CD69, (B) CD98 and (C) 98TM expressing cells labelled with P-Fak, (green) and the cytoskeleton (red).

4.4 DISCUSSION

There is now increasing evidence that CD98 has oncogenic potential. Most tissues, cell lines and tumours appear to express the heavy chain of CD98 (Parmacek et al., 1989; Nakamura et al., 1999). Hara et al., (1999), found that NIH3T3 cells transfected with human CD98 were capable of anchorage independent growth and CD98 transfected clones also gave rise to tumours in athymic mice. Rintoul et al., (2002) has also shown that cross-linking CD98 using the monoclonal antibody 4F2 promoted transformation in SCLC cells. In this study, the structure/function relationship between CD98, intracellular signalling and transformation was examined.

A fundamental feature of anchorage independence is the ability of cells to grow in semi-solid agarose medium. The agar suspension technique uses these properties to clone transformed cells (Sanders and Burford., 1964; Stoker et al., 1968). Anchorage independence reflects the tendency of tumour cells to survive and grow in inappropriate locations *in vivo*, metastasise and invade. This correlates with tumorigenicity and the invasiveness of the tumour (Carney et al., 1980).

Oncogenes are points on normal growth regulatory pathways that are constitutively activated by over expression or mutation. We found that CD98 transfected CHOK1 cells were capable of both anchorage and serum independent growth. Full oncogenic transformation is believed to require both anchorage and serum independent growth. This finding suggests that CD98 mediates oncogenic transformation.

The ability to activate signalling pathways downstream of integrins and integrin activated tyrosine kinases is an important property of oncogenes. Two major cell survival and growth signalling pathways downstream of tyrosine kinase are the PI3-Kinase-AKT pathway and the MAPK pathway (Wei et al., 2001). It has been shown previously that tyrosine kinase activation mediates CD98-induced homotypic aggregation of haematopoietic cells (Warren et al., 1996).

PI3-Kinase catalyzes the phosphorylation of inositol lipids resulting in the formation of 3-phosphorylated phosphoinositides (3-PPI's): [PI(3)P], [PI(3,4)P₂], which generates [PI(3,4,5)P₃]. AKT possesses a PIP₂/PIP₃ binding PH domain, which requires

PI3K stimulation for activation (King et al., 1997). Once activated AKT, can inhibit apoptosis by a number of actions, including the phosphorylation and inactivation of the pro-apoptotic Bcl 2 homolog; Bad, caspase 9 (apoptosis initiating enzyme) and the forkhead family transcription factor FKHR1 (mediates transcription of pro-apoptotic gene products) (Madge et al., 2000). A number of oncogenes and tumour suppressor genes that function upstream of AKT have been found to influence cancer progression by regulating AKT (Datta et al., 2001). Oncogenes which increase PI3-Kinase, eg. Ras and Bcr/Abl have been shown to promote oncogenesis. Khwaja et al., (1997) showed that oncogenic ras conferred cells with anchorage independence by activating the PI3-Kinase and AKT pathway. In 1998, Moore et al., found that PI3-Kinase acting through PKB promoted anchorage independence. By cross-linking CD98 in SCLC cells, Rintoul et al., (2002) showed that PI3-Kinase activation was promoted. In addition to the level of CD98 cell surface expression we found that stimulation of PI3-Kinase activity also affected anchorage independent growth. The level of CD98 cell surface expression affecting anchorage independence may be a consequence of its regulation throughout the cell cycle. CD98 is rapidly up regulated early in transition from G0 to G1 phase, it then remains at elevated levels until the cell cycle is complete (Azzarone et al., 1985; Suomalainen, 1986; Parmacek et al., 1989). Moore et al., (1998) have shown that PI3-Kinase inhibition by LY294002 inhibits SCLC growth, which caused a cell cycle arrest at G1 and a decreased number of cells entering mitosis. Over-expression of CD98 heavy chain stimulated PI3-Kinase activity and CD98 induced anchorage independence was sensitive to PI3-Kinase inhibitors LY294002 and wortmannin (results not shown). This suggests that PI3-Kinase activation is central to the ability of CD98 to regulate cellular activation, progression through G1 phase of the cell cycle and the promotion of anchorage independence.

The results of this study support the hypothesis that there is a threshold level of expression for CD98 mediated transformation as endogenous CD98 does not result in a malignant phenotype. This suggests that constitutively high levels of CD98 expression as found on tumour cells results in PI3-Kinase activation and the promotion of tumorigenesis.

The association of CD98 and $\beta 1$ integrin which is investigated in the next chapter has been shown to occur in the context of low density membranes. Kolesnikova et al., (2001) found that CD98 was localized to the “light” membrane fractions, typically containing caveolae and membrane rafts. These fractions are thought to be enriched with active signalling complexes. However less than 5% of total $\beta 1$ integrins were found in these “light” membrane fractions. It was proposed that the CD98 heavy chain may interact with the amino acid transport light chain and regulate integrins. By mutating the extracellular cysteines in the CD98 heavy chain this interaction was examined. The C109S mutant which disrupts the disulphide linkage to the CD98 light chain showed no association with $\beta 1$ integrins and lost its ability to transform cells. However Fenczik et al., (2001) found that mutation of the cysteines C109S and C330S disrupted the covalent association with the light chain but did not impair interactions or effects on integrins. Even though this covalent association is lost, Pfeiffer et al., (1998) have reported that the C109S mutant still supports the surface expression of the light chain. This therefore indicates that a non-covalent interaction may still be present. The effects of the CD98/CD69 chimeras, especially those which lacked the extracellular domain were examined in order to determine the possibility of a non-covalent interaction between the light and heavy chain of CD98.

Chimeras, in which the transmembrane domain of CD98 heavy chain was replaced with that of CD69, lost the capacity to stimulate PI3-Kinase and promote anchorage independence. However these chimeras had no significant effect on the amino transport function of CD98 (W.Wong data unpublished). When the extracellular domain of CD98 was exchanged with that of CD69, transformation and activation of PI3-Kinase were not affected. These findings indicated that the covalent association of CD98 heavy chain with the light chain is not required for the functional regulation of integrins. These results have also shown that the transmembrane domain of the CD98 heavy chain is both necessary and sufficient for activating PI3-Kinase, elevating intracellular PIP₃ levels and the induction of anchorage independent growth. In addition the transmembrane domain of the CD98 heavy chain was sufficient to promote extensive focal adhesion complexes and to induce these phenotypic changes. In particular by analysing specifically the

chimera 98TM and the mutants Δ 4F2 and D5, the amino acids 82-86 appeared to be critical for these processes.

There are many conflicting views on the subject of cytoskeletal changes in terms of oncogenic potential. As mentioned earlier, a common change in cancer cells is a loss of anchorage dependence and growth control. Boyd et al., (1995) suggested that cytoskeletal components may play a role in the uncoupling of cell shape and growth control. However an intact actin cytoskeleton has also been proposed as an essential part of signal transduction and integrin dependent focal adhesion (Bershadsky et al., 1996). A model used to study integrin activation and function; the homotypic aggregation assay (Bernarczyk et al., 1993) has shown that an active metabolic process and intact cytoskeleton are required for homotypic aggregation (Bednarczyk and McIntyre, 1990). However cytoskeletal changes are required for interactions mediated by specific integrins and signalling proteins (Miyamoto et al., 2003). This observation may explain our findings that in CHOK1 cells over-expressing CD98, the actin cytoskeleton appeared to be intact and that other cytoskeletal components such as microtubules are also unaffected.

The data presented here confirms that the CD98 heavy chain is an oncogene which promotes anchorage and serum independent growth when over expressed in CHOK1 cells. This oncogenic activity is dependent on PI3-Kinase activation and the level of CD98 cell surface. The transmembrane domain, particularly the amino acids 82-86 are essential for activation of PI3-Kinase, elevation of intracellular PIP3 levels, phenotypic changes and anchorage independence. In addition to this, in agreement with Miyamoto et al., (2003), we have found that CD98 heavy chain over-expression has no effect on the actin cytoskeleton and the microtubule network despite being able to promote transformation and PI3-Kinase activity.

This work has been accepted for publication in the Journal of Biological Chemistry and is in press.

CHAPTER 5

INTERACTION OF CD98 WITH $\beta 1$ INTEGRIN

5.0 INTRODUCTION

A requirement for growth and survival of cells is adhesion to extracellular matrix proteins (ECM). This was first described by Stoker et al, (1968) who found that normal cells were blocked in the G1 phase of the cell cycle when cultured in suspension. They termed this phenomenon “anchorage dependence”.

Integrins are a large family of heterodimeric cell surface adhesion receptors which bind ECM proteins and cell surface ligands (Fernandez et al., 1998). These adhesive properties can be regulated by altering their active state through conformational changes or receptor clustering. Integrin signalling can be controlled bi-directionally, “inside out signalling” or “outside in signalling”. The former is initiated by interaction of specific intracellular proteins with the cytoplasmic tail of the integrin promoting a structural change within the integrin. “Outside in signalling” occurs when integrins cluster at distinct sites, known as focal contacts providing a link between the ECM and the actin cytoskeleton, regulating ligand binding. This ability to bind to various proteins allows integrins to play a major role in inflammation, cell adhesion, migration, proliferation and cell differentiation.

Central to integrin function are the integrin β subunit cytoplasmic domains. Chimeric receptors containing β cytoplasmic domains connected to extracellular reporters such as the IL-2 receptor extracellular domain (Tac) or the CD4 extracellular domain can activate specific signalling events such as the phosphorylation of the tyrosine kinase pp125^{FAK} (Akiyama et al., 1994; Lukashev et al., 1994). These domains have also been shown to function as dominant negative mutants, by inhibiting endogenous integrin function by interacting with cytoplasmic components which are crucial for processes

such as cell spreading and cell migration (LaFlamme et al., 1994). Therefore these cytoplasmic domains may mediate integrin function by binding to specific cytoskeletal and signalling proteins which would normally activate endogenous integrins. These include Cytohesin-1, a guanine nucleotide exchange protein which induced α L β 2 dependent binding to ICAM-1 when overexpressed (Kolanus et al., 1996), ILK which was found to bind to β 1 cytoplasmic domains but could also interact with β 2 and β 3 cytoplasmic domains (Hannigan et al., 1996) and signalling proteins such as IAP, a thrombospondin-1 receptor which has been implicated in the modulation of integrin function in β 2 mediated neutrophil migration (Gao et al., 1996, Cooper et al., 1995).

Proteins which interact directly with integrin cytoplasmic domains are potentially excellent candidates for regulators of activation (Hughes and Pfaff., 1998). Recently CD98 has been implicated as a regulator of integrin activation. Fenczik et al., (1997), carried out a genetic cloning strategy based on changes in integrin affinity to identify potential modulators of integrin activation. When isolated integrin β 1 cytoplasmic domains were overexpressed, integrin signalling was suppressed. Using a CHO-cell cDNA expression library and Tac β 1 (overexpression of isolated cytoplasmic β 1 domains) reversal of this dominant suppression was examined. A single cDNA, 5F8 was found to have the ability to reverse Tac β 1 suppression. Sequencing and using a BLAST database showed that the amino acid sequence of 5F8 was 72% identical to the heavy chain of CD98, 4F2. CD98 was found to rescue the suppression of integrin activation caused by Tac β 1. It was also shown that CD98 was involved in the regulation of SCLC adhesion. When CD98 was crosslinked using the monoclonal antibody 4F2, the adhesion of the SCLC cell line H345 to laminin and fibronectin was markedly enhanced. Chandrasekaran et al., (1999) showed that the adhesive and chemotactic activities of thrombospondin-1; an extracellular matrix glycoprotein, could be mediated by CD98. Ligation of CD98, increased $\alpha_3\beta_1$ mediated adhesion of breast carcinoma cells to thrombospondin and to laminin, indicating a role for CD98 in β 1 dependent cell adhesion.

There have been many papers which indicate that CD98 may be involved in integrin function. Anti-CD98 monoclonal antibodies have been demonstrated to activate $\beta 2$ integrins, stimulating monocyte cell-cell aggregation. The subsequent activation of $\beta 1$ integrin leads to cell fusion and the formation of polykaryon, (multinucleated giant cells) (Tabata et al., 1994). This phenotype is seen in many chronic inflammatory diseases such as tuberculosis and sarcoidosis. The link between CD98 and cell fusion has also been investigated, Ohgimoto et al., (1995), demonstrated that FRP-1 (Fusion Regulatory Protein) was an identical molecule to 4F2/CD98. This molecule was found to induce integrin dependent HIV gp160 mediated cell-fusion and to promote cell fusion induced by the Newcastle disease virus (Ito et al., 1992).

Zent et al., (2000) reported that CD98 associated with integrin β cytoplasmic domains with a unique integrin class and splice variant specificity. They found that CD98 interacted with β_{1A} but not β_{1D} or β_7 leukocyte specific cytoplasmic domains. Merlin et al., (2001) found that CD98 selectively immunoprecipitated with LAT-2 and $\beta 1$ and were all found to be polarized to the same (basolateral) domain. This association between CD98 and $\beta 1$ has been further demonstrated by Kolesnikova et al., (2001). They showed CD98 constitutively and specifically associated with a variety of $\beta 1$ heterodimers. Rintoul et al., (2002) has also shown that CD98 constitutively associated with $\beta 1$ regardless of activation state in SCLC.

The aim of this part of the study was to investigate this interaction further and determine which part of the CD98 molecule was important for this association using the CD98/CD69 chimeras discussed in chapter 3.

5.1 Optimisation of immunofluorescence conditions :

5.1.1 The 9EG7, rat monoclonal antibody gave optimal staining of the $\beta 1$ integrin.

In order to examine the expression of $\beta 1$ integrin on CHOK1 cells stably transfected with either CD98 or CD69 or the chimeras, a number of $\beta 1$ integrin antibodies were tested using the secondary antibodies, alexa fluor 568. Eight different antibodies were tested, 9EG7, 12G10, N-20, TS216, K20, MCA1949, Chemicon $\beta 1$ and BD $\beta 1$, however the three most effective are shown in Figure 5.1.1. In comparison to TS216, K20 and the other five antibodies tested, 9EG7 stained the membrane exclusively. This staining was in a punctate fashion which had been previously described by Rintoul et al., (2002), when they investigated $\beta 1$ colocalisation in SCLC using K20, and TS216.

Therefore 9EG7 was the $\beta 1$ antibody used throughout this investigation as this was the most specific to $\beta 1$ integrins in hamster cells.

5.1.2 Localisation of $\beta 1$ integrin within the cell membrane.

The 9EG7 rat monoclonal antibody recognises an epitope up-regulated in response to Mn^{2+} (Lenter et al., 1993). $\beta 1$ integrin function is inhibited by Ca^{2+} because it prevents a conformation favourable to ligand binding. However, Mn^{2+} enhances $\beta 1$ function inducing the same active conformation that is formed by adding ligand or removing Ca^{2+} , (Bazzoni et al., 1995). It has been demonstrated in previous studies that $\alpha_5\beta_1$ mediated cell adhesion can be enhanced by Mn^{2+} (Bazzoni et al., 1995), and this stabilises the $\beta 1$ conformation recognised by the 9EG7 antibody (Lenter et al., 1993, Mould et al., 1998). This suggests that the integrins are in an active conformation which promotes cell attachment, when they bind 9EG7 (Mastrangelo et al., 1999). 9EG7 is therefore a useful antibody to study regulation of $\beta 1$ integrins, as it identifies activated $\beta 1$.

Ng et al (1999) found that in untreated MCF-7 cells activated $\beta 1$ integrin was found predominantly in a perinuclear/pericentriolar compartment where $\beta 1$ recycles. However when the cells were treated with a PKC activator, the $\beta 1$ integrin redistributed to the plasma membrane. Equally a dramatic redistribution of activated $\beta 1$ to the plasma membrane was observed after a short incubation at 20°C. They hypothesised that $\beta 1$ is being constantly recycled to the cell surface, a process which is abolished at low temperatures. Using the technique described in MATERIALS AND METHODS, CHOK1 cells stably transfected with CD98, were fixed using 3% PFA for 20 minutes and then incubated with 9EG7, post fixation), (Figure 5.1.2 (A)). This localised $\beta 1$ within the perinuclear region of the cell. However when cells were incubated with 9EG7 at 4°C before fixation with 3% PFA for 20 mins, $\beta 1$ localised within the plasma membrane, (pre-fixation), (Figure 5.1.2. (B)).

These findings corroborate with those of Ng et al., (1999) and imply that there is constant trafficking of integrin to the cell surface and that $\beta 1$ is being continuously recycled.

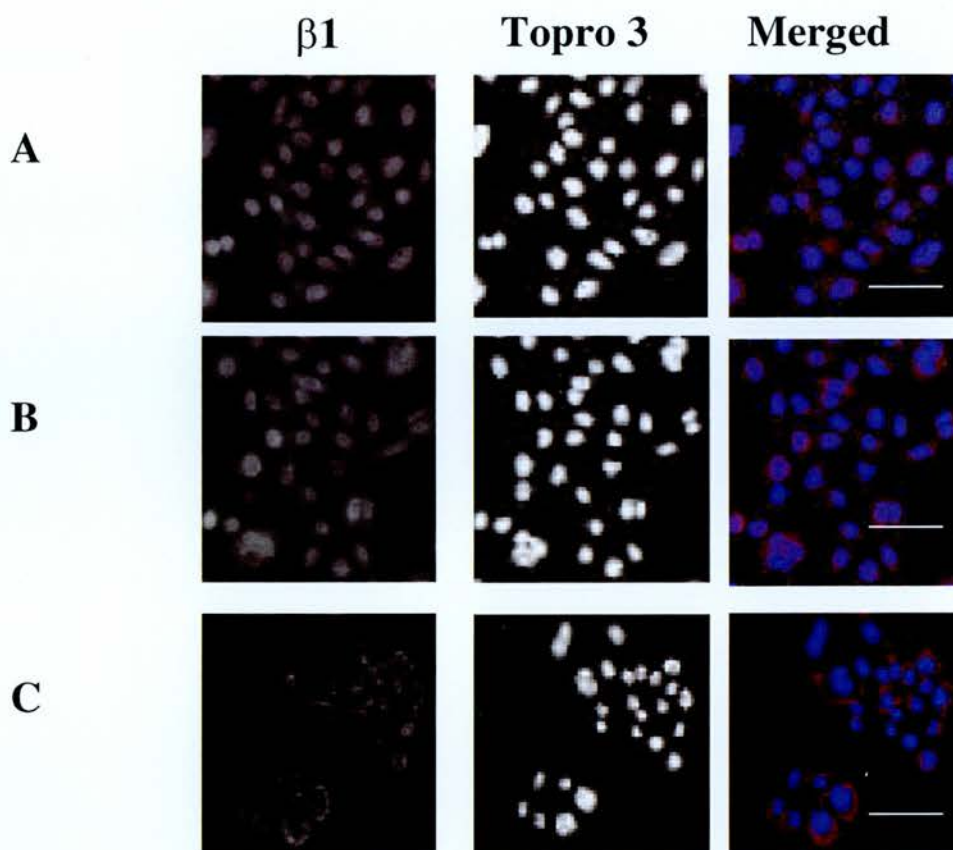


Figure 5.1.1 Binding of $\beta 1$ integrin antibodies to CHOK1 cells stably transfected with CD98.

Stable CHOK1 cells were incubated with various antibodies against $\beta 1$ integrin, as described in MATERIALS and METHODS. White bars 38 μ m Confocal microscopy was performed with a TCS NT Confocal microscope (Leica). Localisation of $\beta 1$ integrin using (A) TS216 (red), (B) K20 (red) and (C) 9EG7 (red) and corresponding secondary antibody alexa fluor 568 in a single confocal plane. The nucleus was visualised using TOPRO 3 (blue). TS216 and K20 gave perinuclear staining whereas the 9EG7 antibody specifically stained the plasma membrane. The images shown here are representative views of 3 independent experiments.

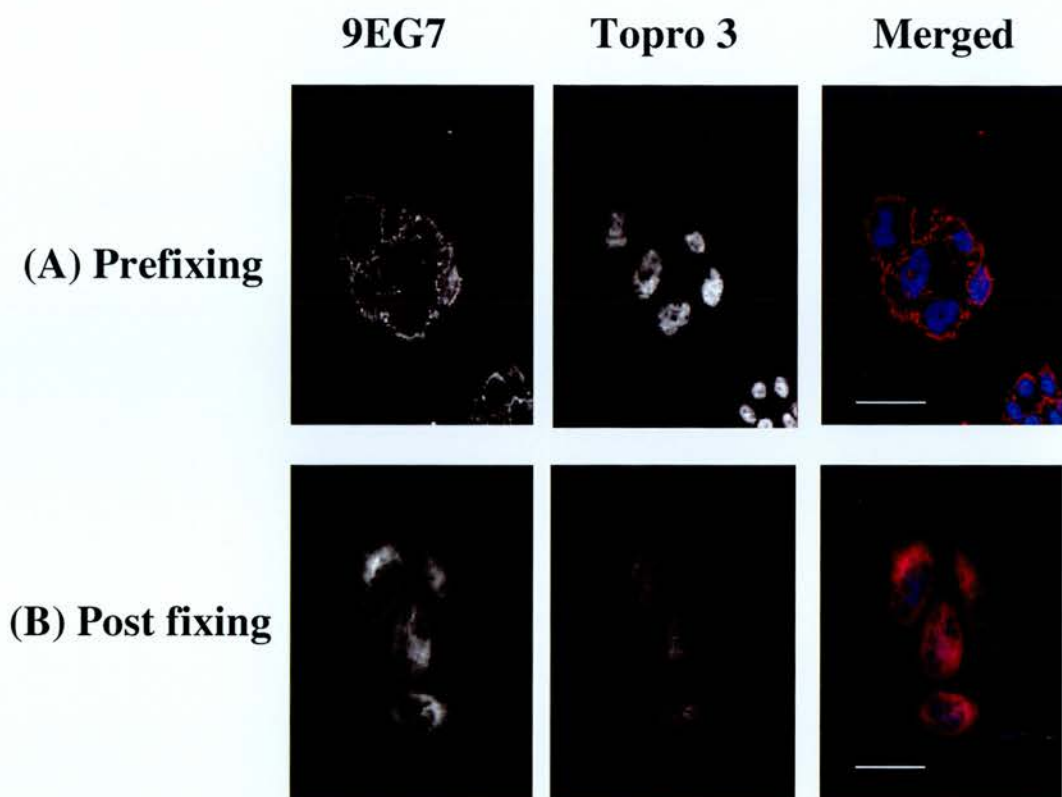


Figure 5.1.2 Optimising fixation conditions in CHOK1 cells stably transfected with CD98.

Stable CHOK1 cells were incubated with antibodies against $\beta 1$ integrin (9EG7), (A) pre (labelling with 9EG7 was performed at 4°C before fixing with 3% PFA and then the cells were incubated with the secondary alexa fluor 568) or (B) post fixation, (labelling with 9EG7 and then the secondary alexa fluor 568 after fixation with 3% PFA). Images were taken using a TCS NT Confocal microscope (Leica). Localisation of $\beta 1$ integrin using 9EG7 (red) in a single confocal plane. Nuclear staining was visualised using TOPRO3 (blue). Staining with 9EG7 post fixation gave perinuclear staining whereas labelling pre fixation appeared to give clear $\beta 1$ labelling around the plasma membrane. White bars, 11.7 μ m. The images shown here are representative views of 3 independent experiments.

5.1.3 Optimisation of fixation conditions.

Stable CHOK1 cells were incubated with 9EG7 at 4°C and then fixed using three different fixation techniques, described in MATERIALS AND METHODS, (Figure 5.1.3). Methanol fixation works by precipitating proteins. With this fixation there is no need to permeabilise the cells or quench the fixative. However lipids and soluble proteins will be extracted from the cell and the morphology of the cell is significantly changed with the cell becoming thin. Compared to fixation with 100% methanol and fixation using 50:50, Methanol:Acetone, fixation with 3% PFA gave the optimal images. (Figure 5.1.3 (C)), 3% PFA gave very clean and clear labelling of the cells and allowed permeabilization of the cell membrane while retaining the overall architecture of the cells.

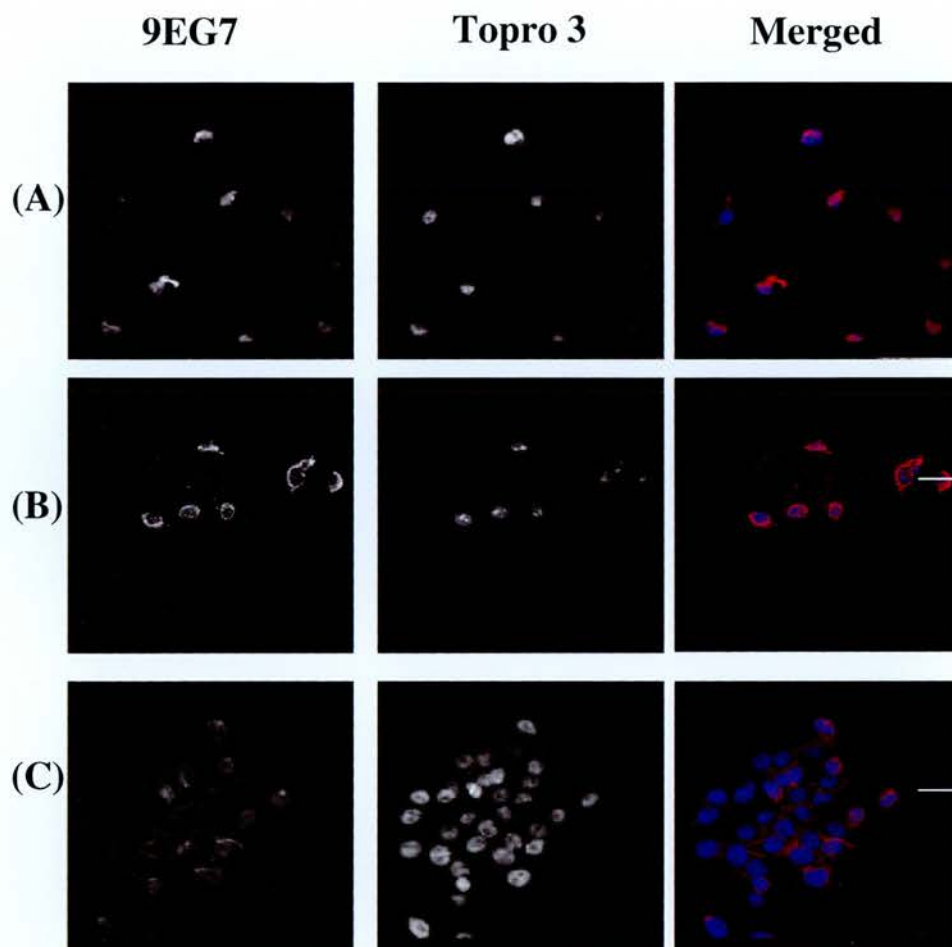


Figure 5.1.3 Comparison of fixation methods in CHOK1 cells stably transfected with CD98.

Stable CHO K1 cells were incubated with 9EG7 at 4°C and then fixed. The cells were then incubated with a secondary antibody alexa fluor 568. Localisation of β 1 integrin using 9EG7 (red) in cells fixed with:- (A) 100% methanol at -20 °C for 10 minutes, (B) 50:50, methanol:acetone at -20°C for 30 seconds or (C) 3% PFA. Nuclear staining was visualised using TOPRO3 (blue). White bars 23.6 μ m. Images were taken using a TCS NT confocal microscope (Leica). Both fixation with 100% methanol and 50:50 methanol:acetone gave unspecific staining and was located in the perinuclear region. However fixation with PFA gave clean and clear staining in a punctuate fashion around the plasma membrane. Shown here are representative images from 3 independent experiments.

5.2 Colocalisation of CD98 and β 1 integrins

5.2.1 CD98 colocalises with β 1 integrins.

Rintoul et al (2001) showed that β 1 and CD98 were constitutively associated in SCLC cells using immunofluorescence. Thus this interaction was further examined to determine which part of the CD98 molecule was important for this association.

The relationship between β 1 integrins and CD98 was examined in CHOK1 cells stably transfected with CD98 or CD69 or the CD98 chimeras detailed in Chapter 2 by dual label confocal immunofluorescence. In the CD98 stably expressing cells, CD98 (detected by 4F2) and the β 1 integrin (detected by 9EG7) were colocalised in the plasma membrane. However, no colocalisation was seen between CD69 (detected by FN50-FITC) and β 1 in the CD69 stables. The chimeras 4F2TM which has the extracellular domain of CD98 replaced with that of CD69 and 4F269 which has the extracellular and transmembrane domain replaced with those of CD69 were examined. Colocalisation was seen in cells expressing 4F2TM but not in cells expressing 4F269, (Figure 5.2.1a). 69TM a chimera which has its transmembrane domain replaced with CD69 did not show colocalisation between β 1 and CD98 whereas 69cyto which has its cytoplasmic domain replaced with that of CD69, did show colocalisation, (Figure 5.2.1b). The 98TM chimera which has the extracellular and cytoplasmic domain of CD69 and the transmembrane of CD98 and cells expressing the chimera 98EX which has the transmembrane and cytoplasmic domain of CD69 and the extracellular domain of CD98 showed colocalisation of CD98 and β 1, (Figure 5.2.1c). The truncation mutant Δ 4F2 which only has the extracellular domain and transmembrane domain of CD98 also showed colocalisation, (Figure 5.2.1d). The isotype controls, IgG₁ and IgG_{2A} antibodies for CD98 /CD69 and β 1, respectively, showed no evidence of non-specific binding.

These findings indicate that the transmembrane domain of CD98 is necessary and sufficient for colocalisation with β 1 integrins. However the finding that cells expressing 98EX colocalised also indicate an importance for the extracellular domain of CD98.

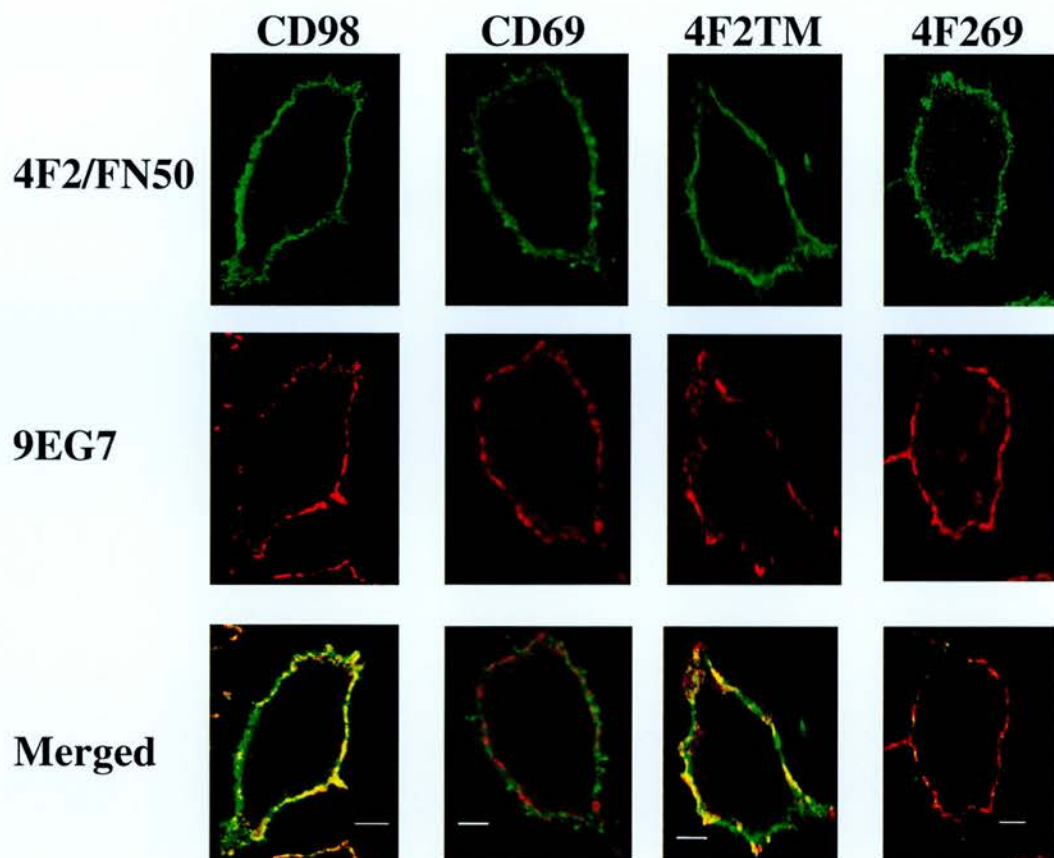


Figure 5.2.1a β 1 integrin and CD98 are colocalised in CHOK1 cells stably transfected with CD98 and the CD98/CD69 chimera 4F2TM.

Stable CHOK1 cells were incubated with 9EG7 and 4F2 or FN50 as described in MATERIALS and METHODS. White bars, 5 μ m. Localisation of either CD98 or CD69, is shown in the upper panels using either 4F2 or FN50 respectively). β 1 localisation is shown in the middle panel using 9EG7. In the merged panel, areas of colocalisation of β 1 and CD98 appear yellow. All images shown are of a single confocal plane. Both CHOK1 cells stably expressing CD98 and the chimera 4F2TM showed colocalisation between β 1 and CD98 whereas the cells stably expressing CD69 and the chimera 4F269 did not. Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica). Images were analyzed using Bitplane Imaris 4 software. Images shown are representative of 5 independent experiments.

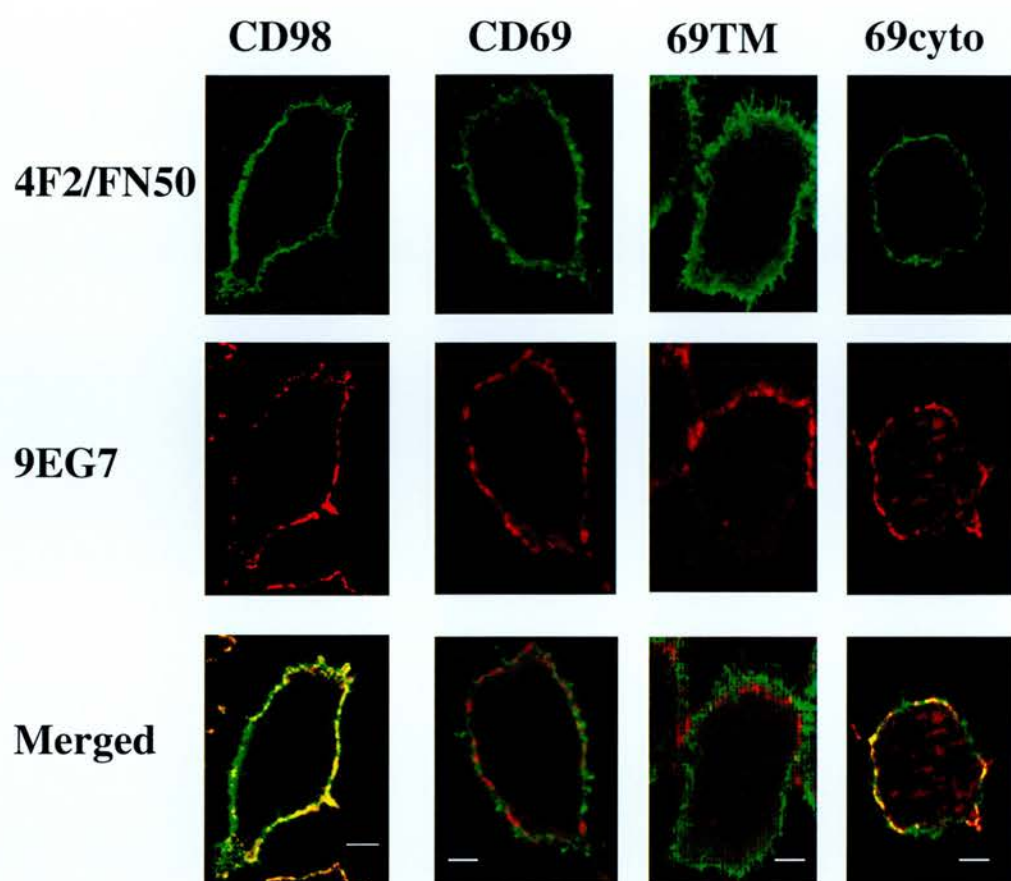


Figure 5.2.1b β 1 integrin and CD98 are colocalised in CHOK1 cells stably transfected with the CD98/CD69 chimera, 69cyto.

Stable CHO K1 cells were incubated with 9EG7 and 4F2 (CD98) or FN50 (CD69) as described in MATERIALS AND METHODS. White bars 5 μ m. Localisation of either CD98 or CD69 is shown in the upper panels using either 4F2 or FN50 respectively. β 1 localisation is shown in the middle panel using 9EG7. In the merged panel, areas of colocalisation of β 1 and CD98 appear yellow. All images shown are of a single confocal plane. CHOK1 cells stably transfected with the chimera 69cyto showed colocalisation of β 1 and CD98 similar to that seen with CD98 stably expressing cells. The cells stably expressing the chimera 69TM did not show colocalisation. Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica). Images were analyzed using Bitplane Imaris 4 software. Images shown are representative of 5 independent experiments.

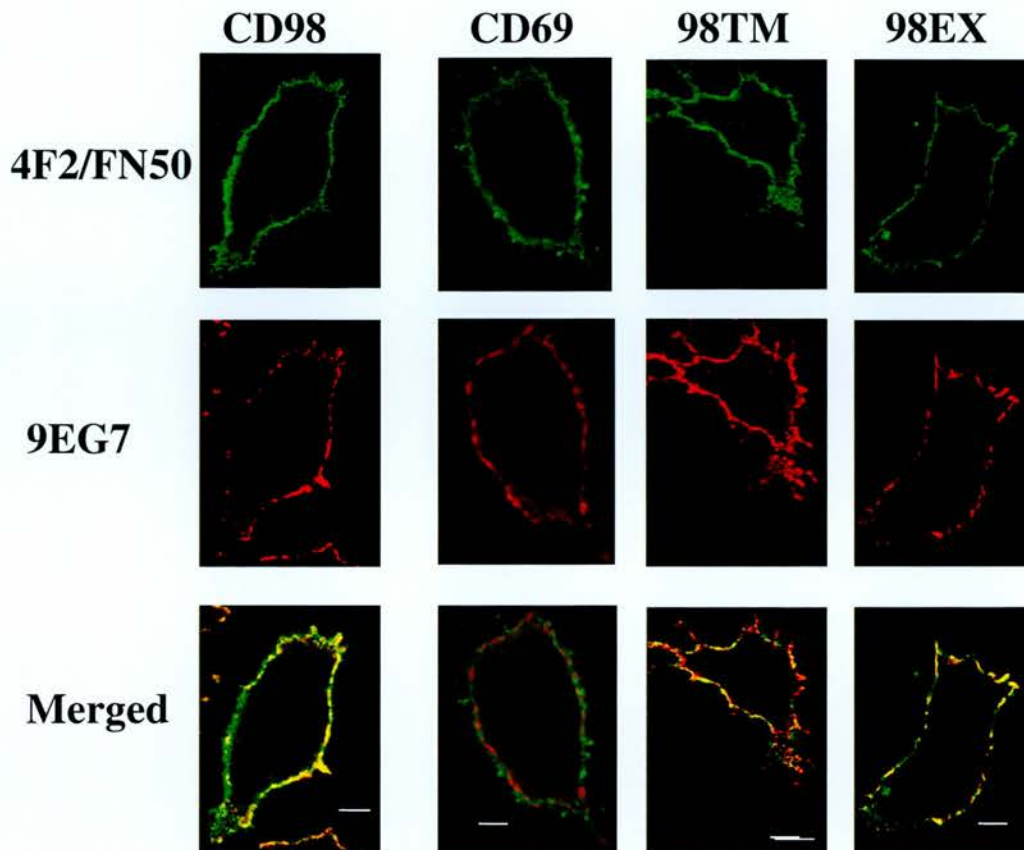


Figure 5.2.1c β 1 integrin and CD98 are colocalised in CHOK1 cells stably transfected with the CD98/CD69 chimeras, 98TM and 98EX.

Stable CHOK1 cells were incubated with 9EG7 and 4F2 (CD98) or FN50 (CD69) as described in MATERIALS AND METHODS. White bars 5 μ m. Localisation of either CD98 or CD69 is shown in the upper panels using either 4F2 or FN50 respectively. β 1 localisation is shown in the middle panel using 9EG7. In the merged panel, areas of colocalisation of β 1 and CD98 appear yellow. All images shown are of a single confocal plane. CHOK1 cells stably expressing the chimeras 98TM and 98EX showed colocalisation of β 1 and CD98, similar to that seen in cells expressing the whole CD98 heavy chain. Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica). Images were analyzed using Bitplane Imaris 4 software. Images shown are representative of 5 independent experiments.

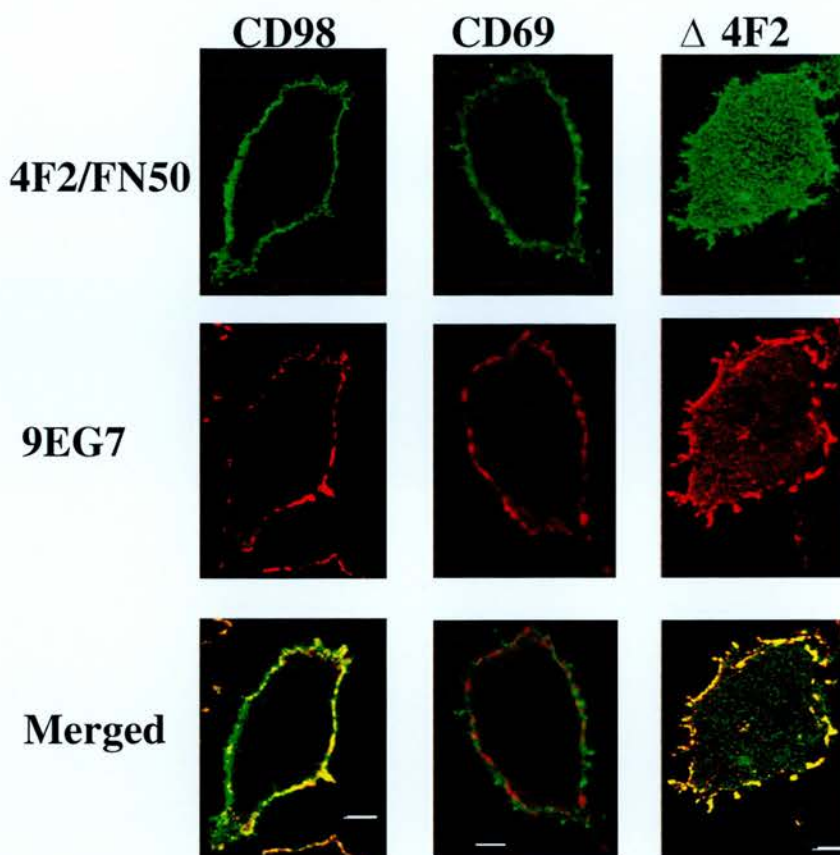


Figure 5.2.1d $\beta 1$ integrin and CD98 are colocalised in CHOK1 cells stably transfected with the CD98 truncation mutant, Δ 4F2.

Stable CHOK1 cells were incubated with 9EG7 and 4F2 (CD98) or FN50 (CD69) as described in MATERIALS AND METHODS. White bars 5 μ m. Localisation of either CD98 or CD69 is shown in the upper panels using either 4F2 or FN50 respectively. $\beta 1$ localisation is shown in the middle panel using 9EG7. In the merged panel, areas of colocalisation of $\beta 1$ and CD98 appear yellow. All images shown are of a single confocal plane. CHOK1 cells stably expressing the mutant, Δ 4F2 showed colocalisation of $\beta 1$ and CD98, especially at the ends of the filopodia. Confocal microscopy was performed using a TCS NT Confocal microscope system, (Leica). Images were analyzed using Bitplane Imaris 4 software. Images shown are representative of 5 independent experiments.

5.2.2 Confocal analysis

To quantify these findings, confocal images were analyzed using Bitplane Imaris 4 software. This software examines the voxels (3D cubes) within these images, which overlap and the programme forms a colocalisation channel. This provides data which can be expressed quantitatively, (figure 5.2.2). The histogram represents the total amount of colocalisation between CD98 and $\beta 1$ in each stable from 3 independent experiments. CHOK1 cells stably transfected with CD98, Δ 4F2, 98TM, 4F2TM and 69cyto, (61.2 ± 8.5 , 79 ± 1.0 , 69.8 ± 5.5 , 62.7 ± 5.0 and 46.5 ± 0.5 respectively), showed significantly higher percentages of $\beta 1$ colocalisation compared to the stables of the negative control, CD69, (26.3 ± 11.20). 98EX gave variable results (44.5 ± 22.5) but 40% of $\beta 1$ integrins colocalised in cells expressing 98EX.

This again confirms the importance of the transmembrane domain of CD98 in colocalisation with $\beta 1$ and indicates a possible role for the extracellular domain of CD98.

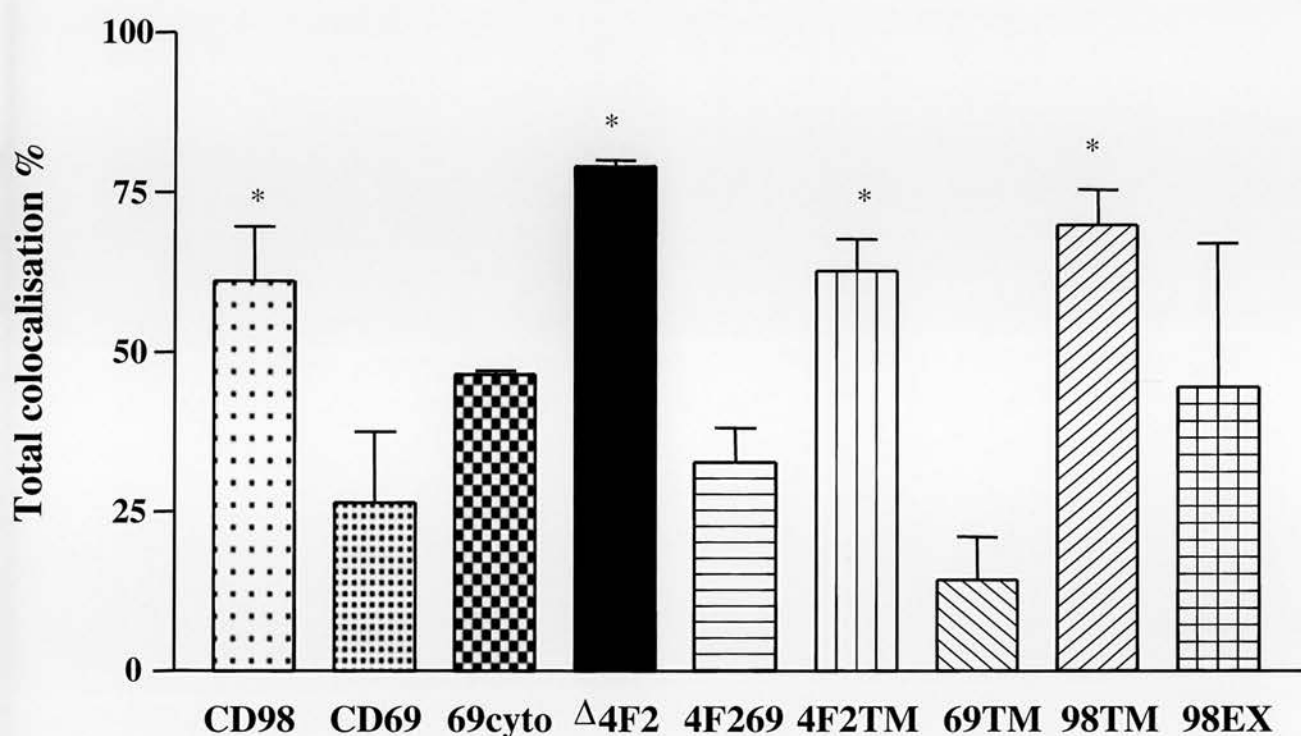


Figure 5.2.2 Colocalisation of $\beta 1$ with either CD98,CD69 or various chimeras.

Confocal images were analysed using Bitplane Imaris 4 software and the % of colocalisation of CD98 with $\beta 1$ was calculated. The results are expressed as % colocalisation and are the mean \pm SEM of 5 independent experiments.

CD69 colocalisation is minimal compared to CD98. The stables which had the transmembrane domain of CD98 also had significantly higher percentages of colocalisation compared to CD69 and 4F269. The cells expressing the chimera 98EX also colocalised with approximately 40% of the $\beta 1$ integrins.*= significant compared to CD69 transfected cells ($P < 0.05$, ANOVA).

5.2.3 Amino acids 82-86 of CD98 are critical for integrin association.

To further examine the interaction of CD98 with $\beta 1$ integrins, the CD98 truncation mutant D5 was used. The D5 mutant lacks amino acids 1-86, but is well expressed in CHOK1 cells, as shown by flow cytometry, (figure 4.3.4). Dual label immunofluorescence was carried out using CHOK1 cells stably transfected with the chimera D5,(figure 5.2.3). While 98TM (which has the extracellular and cytoplasmic domain of CD69 and the transmembrane domain of CD98) and $\Delta 4F2$ (truncation mutant which lacks the putative cytoplasmic tail (amino acids 2-77)), were able to colocalise with $\beta 1$ integrins, the D5 mutant was unable to colocalise. This was confirmed using the Bitplane Imaris 4 software (as above). Figure 5.2.3 (D), represents the total amount of colocalisation between CD98 and $\beta 1$ in each stable. CHOK1 cells stably transfected with CD98 showed significantly higher percentages of $\beta 1$ colocalisation compared to the stables of my negative control, CD69 and the truncation mutant D5.

These results suggest that the five amino acids 82-86 at the cytoplasmic tail/transmembrane domain interface are required for integrin association and are in agreement with work done by Cal et al., (2005). They have also showed that when these five N-terminal amino acids (WALLL) are deleted no increase in adhesion or migration is seen in IMCD cells compared to the wild type controls. When CD98 is truncated before the tryptophan residue of the WALLL sequence but retains the first five amino acids (WVRTR) of the cytoplasmic tail, these cells adhere and migrate on to collagen to a similar degree as the CD98 expressing cells. Their results indicate that the WALLL sequence is required for increased cell adhesion and migration and is therefore required for CD98- $\beta 1$ interactions.

The transmembrane domain and juxtamembrane portion of the cytoplasmic tail of CD98 has been shown to be 100% identical in all known mammalian CD98 orthologs highly conserved in other CD98 homologs (Cal et al., 2005). These findings suggest that these domains play a critical role in mediating specific cellular functions.

5.2.4 Iso-surface rendering of CD98, CD69 and 98TM stables.

Colocalisation images were analysed using Bitplane Imaris 4 software, and 3D images were made of various chimeras (figure 5.2.4). This programme takes into account information about the whole cell. The localisation of $\beta 1$ was on the basolateral surface as seen by Cook et al, (2002) who found that $\beta 1$ colocalised with CD9 (a member of the TM4SF and tetraspanin) in punctuate patches across the lower cell surface, and filopodia. The location of CD98 and CD69 was more generalised across the whole of the cells. In the CHOK1 cells stably transfected with CD69 the $\beta 1$ staining appeared to form a separate layer from that of CD69, whereas in the CD98 stables the CD98 and $\beta 1$ layers appeared to localise together. The colocalisation channel formed using the Biplane Imaris 4 software confirmed these observations. This channel (yellow), (Fig 5.2.4(C)), showed areas within the cell where colocalisation of CD98/CD69 and $\beta 1$ was present. While the stables of CD69 had very little colocalisation, both CD98 and the 98TM stables had significantly more CD98 and $\beta 1$ colocalising. These 3D images also showed that CD98 stable CHOK1 cells were quite flattened and elongated and the stables of 98TM showed the same phenotype. However the CD69 stables had a more rounded morphology.

This analysis indicated that the transmembrane domain of CD98 was sufficient to induce phenotypical changes.

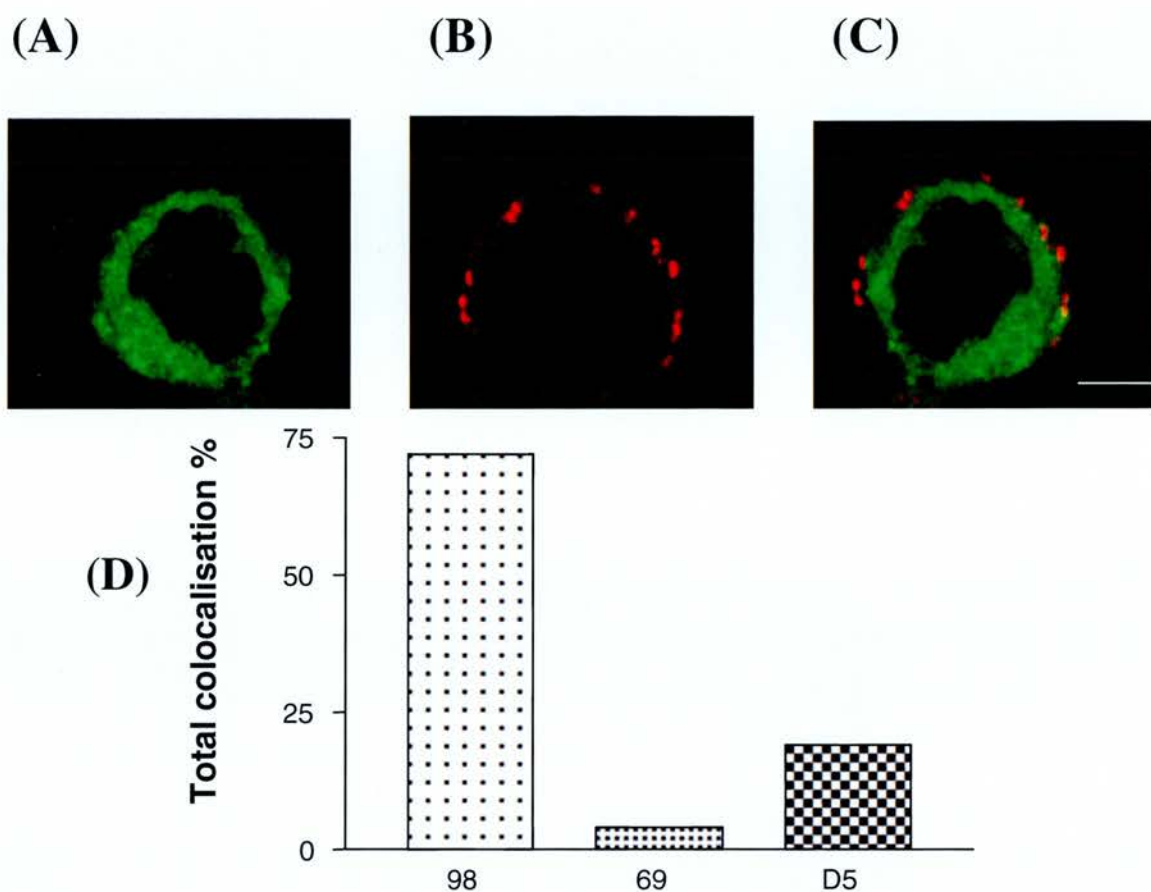


Figure 5.2.3. The chimera D5 shows no colocalisation between $\beta 1$ and CD98.

CHOK1 cells stably transfected with the chimera D5 were incubated with antibodies against $\beta 1$ integrin and CD98 as described in MATERIALS and METHODS. White bars, 5 μm . Images were taken using a TCS NT confocal microscope, (Leica).

(A) Localisation of CD98, (4F2-green), (B) $\beta 1$ integrin (9EG7-red) and (C) merged images. (D) Total colocalisation of CD98 and $\beta 1$ in D5 stably expressing cells compared to those of CD98 and CD69. D5 colocalises with a third of the $\beta 1$ which CD98 does.

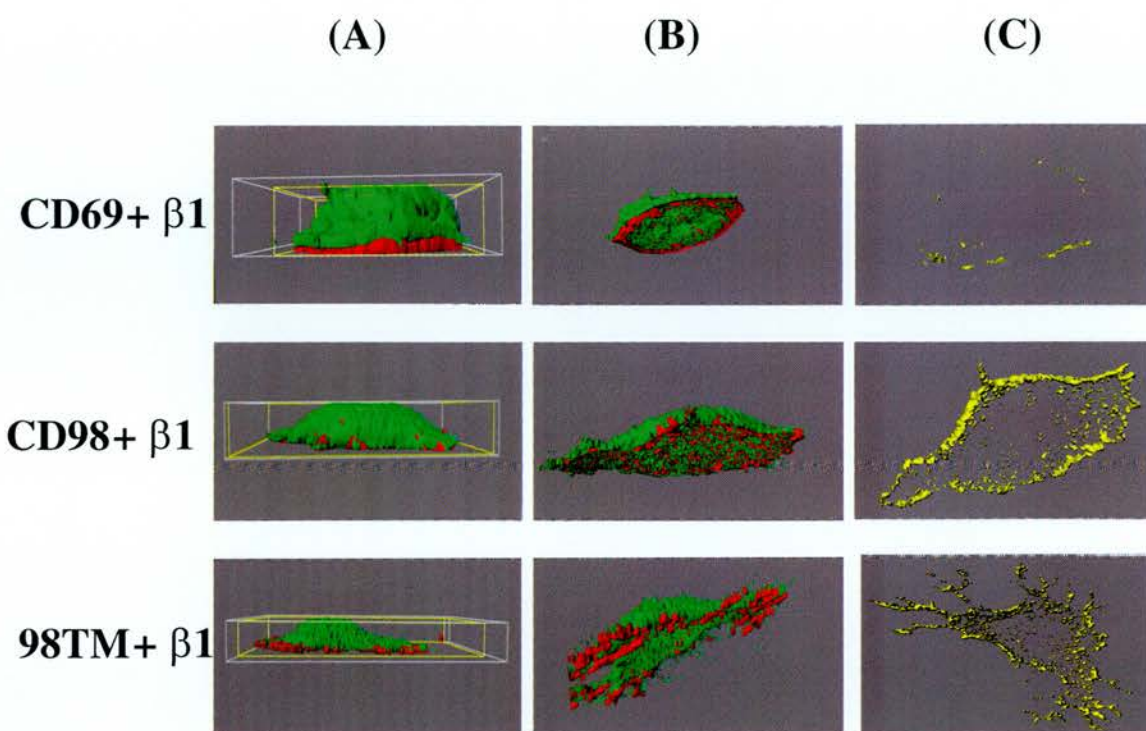


Figure 5.2.4 Iso-surface rendering of CD98, CD69 and 98TM.

Colocalisation images were analysed using Bitplane Imaris 4 software, and 3D images were made of various chimeras. Images (A) and (B) indicate localisation of either CD98 (4F2) or CD69 (FN50) as green and β 1 integrin, (9EG7) as red. (C) The yellow is the colocalisation channel formed using the Imaris software.

5.3 Improving image quality by deconvolution.

Opinions vary on the value of colocalisation and whether it is an accurate measure of physical association between two molecules. This antipathy towards colocalisation is mainly due to the limitations of confocal microscopy in terms of lateral spatial resolution. This is the resolution in the direction of the optical axis, called z, and related to the depth of field which depends on the numerical aperture of the objective lens.

When an image is generated using confocal microscopy the image is convolved, (Figure 5.3.0). This is where every point of the object is replaced by an appropriately blurred point and the final image is the sum of these blurred points. The point spread function (PSF) describes the spreading or blurring of a point source of light under specific optical conditions. Deconvolution is an improved method for confocal imaging as it effectively reverses the convolution process. This process uses the PSF, a mathematical algorithm, which removes the blur and haze from an image. Deconvolution therefore improves the resolution of the image especially along the z axis.

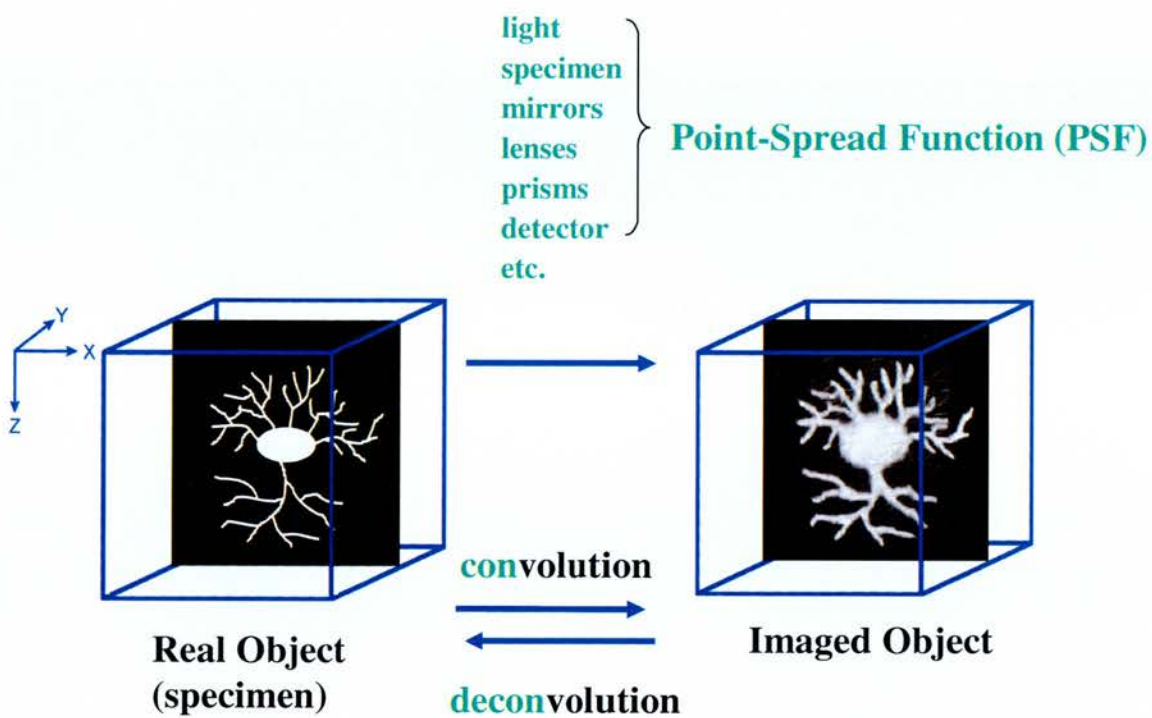
To confirm that the colocalisation seen with the CD98 stables was real and not a consequence of poor resolution, scans were taken of both CD98 and CD69 and these images were then loaded in to Huygens, a deconvolution software package.

Figure 5.3.1 (A) and (C) demonstrate the poor resolution along the z axis which is obtained through confocal microscopy. The resolution was greatly improved when the images of CD98 and CD69 were deconvolved, (B) and (D). The blur had virtually disappeared and the image quality was improved along both the x-z axis and y-z axis.

This improved resolution was again then visible when x-y slices were analysed before and after the deconvolution process, (Figure 5.3.2). This process further confirmed that the colocalisation between $\beta 1$ and CHOK1 CD98 stables was real. Deconvolved images were analysed by the Bitplane Imaris 4 software, as before, (Figure 5.3.3). There was a 2 fold increase in $\beta 1$ colocalisation in CHOK1 cells stably

expressing CD98, compared to cells expressing CD69, (mean \pm SEM, 51.50 ± 2.723 and 26.00 ± 1.080 , respectively).

These experiments demonstrated the value of deconvolution in colocalisation studies and confirmed that CD98 and $\beta 1$ constitutively colocalise in CD98 CHOK1 stables.



Deconvolution figure adapted from Linda Wilson

Figure 5.3.0 The process of deconvolution

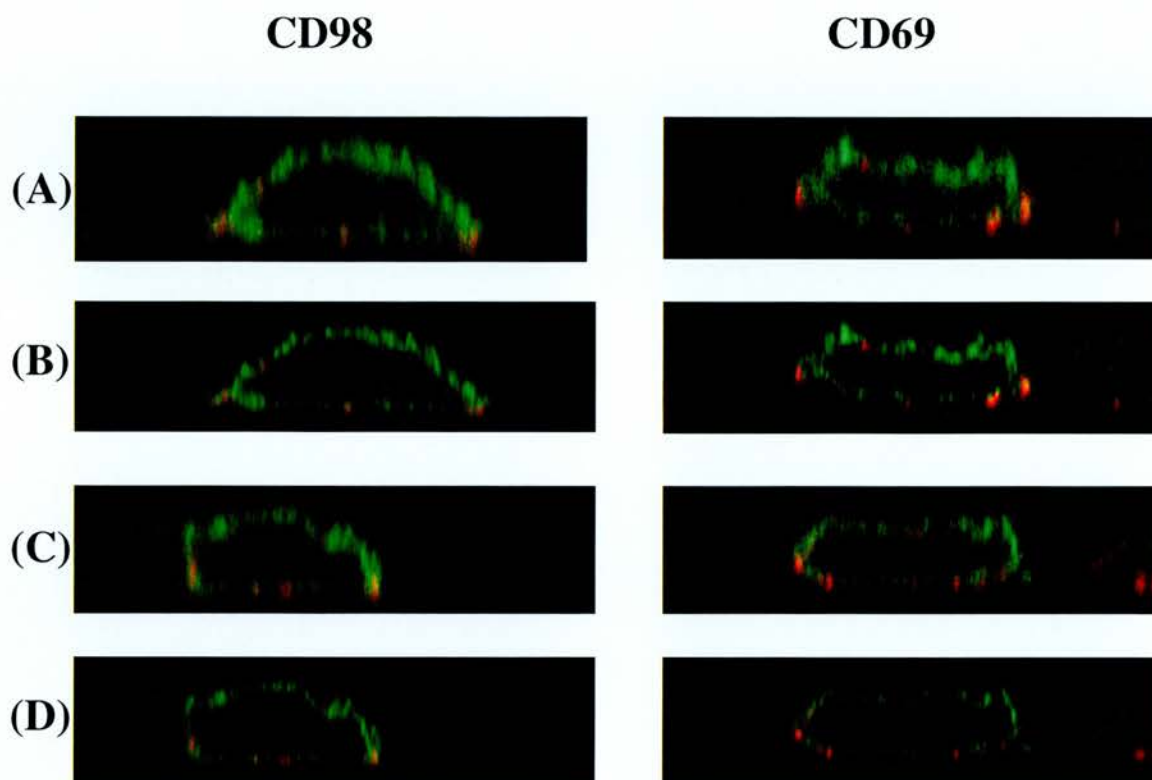


Figure 5.3.1 Deconvolution improves the resolution of the x-z axis and y-z axis.

Images of CD98 and CD69 expressing cells were taken using a TCS NT Confocal microscope system, (Leica) and then deconvolved using *Huygens* software. (A) and (B) are images of a slice taken at the x-z axis. (C) and (D) are images of a slice taken at the y-z axis. (A) and (C) show the original confocal images and (B) and (D) show images after deconvolution.

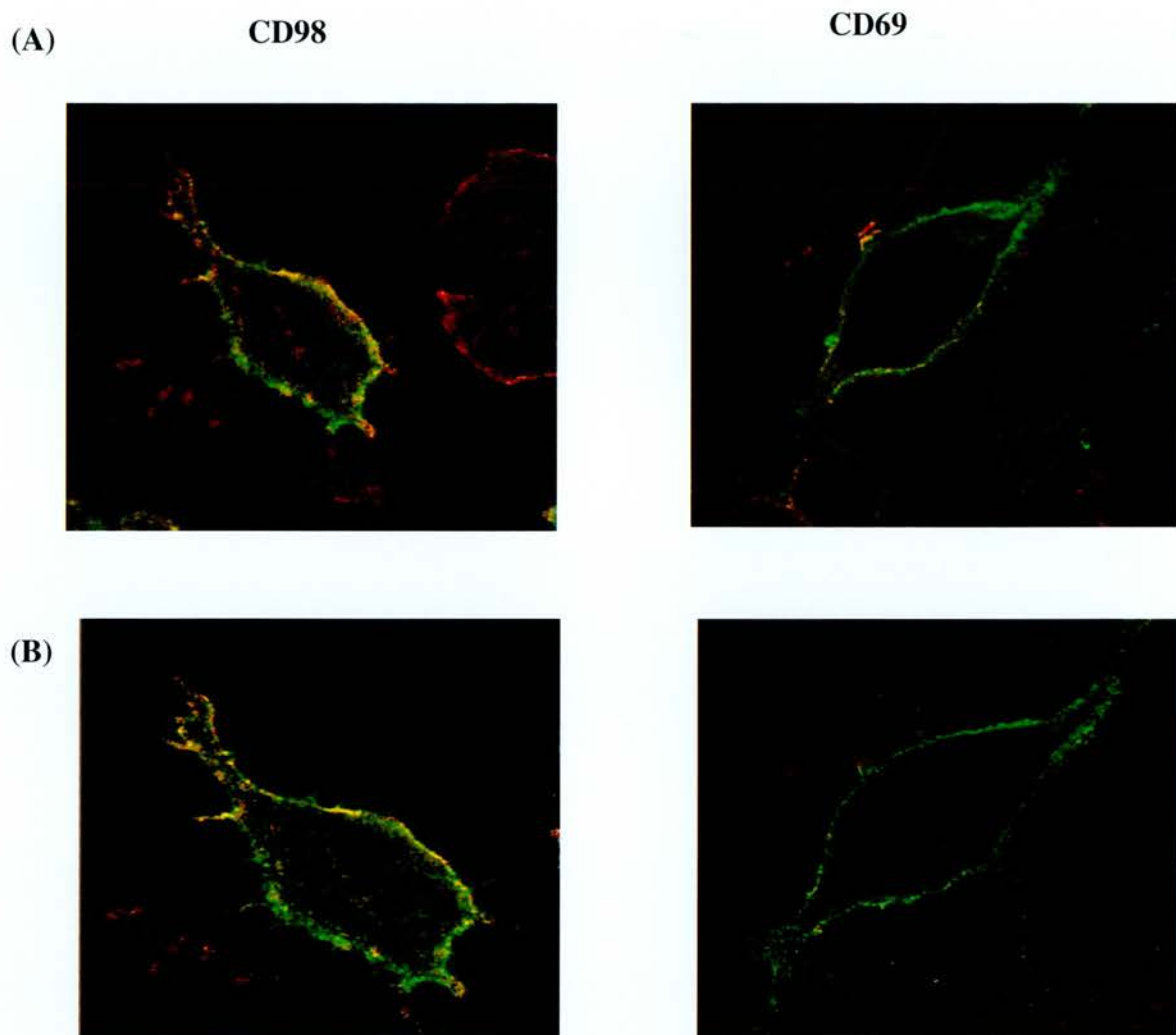


Figure 5.3.2 Deconvolution confirms colocalisation of CD98 with $\beta 1$.

Cells were labelled with 9EG7 and 4F2 or FN50 as described in MATERIALS and METHODS. Confocal microscopy was performed using a TCS NT confocal microscope system, (Leica). Images were deconvolved using *Huygens*. (A) is an image of CHOK1 cells stably transfected with CD98 or CD69 before deconvolution. (B) shows the same images deconvolved. Areas of colocalisation appear yellow. These images are representative of 3 independent experiments.

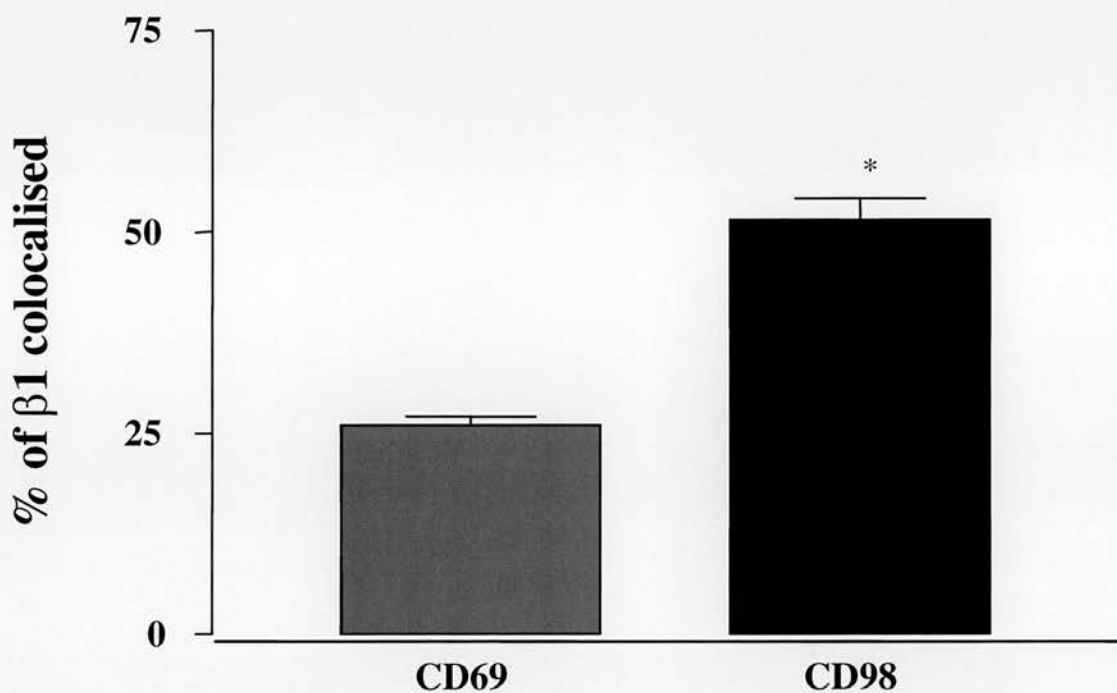


Figure 5.3.3 Colocalisation of $\beta 1$ with CD98 or CD69 after deconvolution.

Colocalisation data was analysed after deconvolution using Bitplane Imaris 4 software. The graph represents the percentage of $\beta 1$ colocalised with either CD98 or CD69 within the whole cell. These results are the mean \pm SEM of 3 independent experiments. Over 50% of $\beta 1$ colocalises with CD98 stably expressing CHO K1 cells after deconvolution compared to the 25% colocalisation seen with CHO K1 cells stably expressing CD69. * = significance = $P < 0.05$ (ANOVA).

5.4 Immunoprecipitation of CD98 and β 1 integrin

5.4.1 The transmembrane domain of CD98 is sufficient to co-immunoprecipitate with β 1.

Zent et al., (2000), showed that CD98 interacted with integrin β cytoplasmic domains which had unique class and splice variant specificity. They found that CD98 associated with β 1A but not the muscle specific splice variant β 1D or the leukocyte specific β 7 cytoplasmic domain. CD98 was also found to selectively immunoprecipitate with both LAT-2 and β 1 integrin and was found to polarize to the same domain, (Merlin et al., 2001). Rintoul et al., (2002) also confirmed these results. I was therefore interested in whether this association occurs in CHOK1 cells stably transfected with CD98 and which part of the CD98 molecule was important for this process.

Protein lysates were made from CHOK1 cells stably transfected with either CD98 or CD69, or the chimeras as described in MATERIALS AND METHODS. CD98, CD69 or β 1 were immunoprecipitated from these lysates, using either (2 μ g) 4F2, FN50 or K20 antibodies respectively. Washed immunoprecipitates were resolved by SDS-PAGE and subsequent western blots were then probed for either CD98, CD69 or β 1. The top panel in Figure 5.4.1 (A) shows that β 1 was immunoprecipitated uniformly from all cells. The lower panel shows that CD98 co-immunoprecipitates with β 1. A band corresponding to CD98 was observed at 80kDa in the CD98 expressing cells and in the high expressing CD98 cells (CD98+) and a truncated form 72 kDa, was seen in Δ 4F2 cells. However in the 69TM expressing cells, which have its transmembrane domain replaced with CD69, CD98 immunoreactivity was not detected.

When the lysates were immunoprecipitated by CD98 or CD69, (figure 5.4.1(B)), β 1 was detected in CD98, CD98+, Δ 4F2, and 98TM expressing cells. However in the CD69 cells and the cells expressing the CD69 transmembrane domain, such as 4F269 and 69TM, β 1 was not detected.

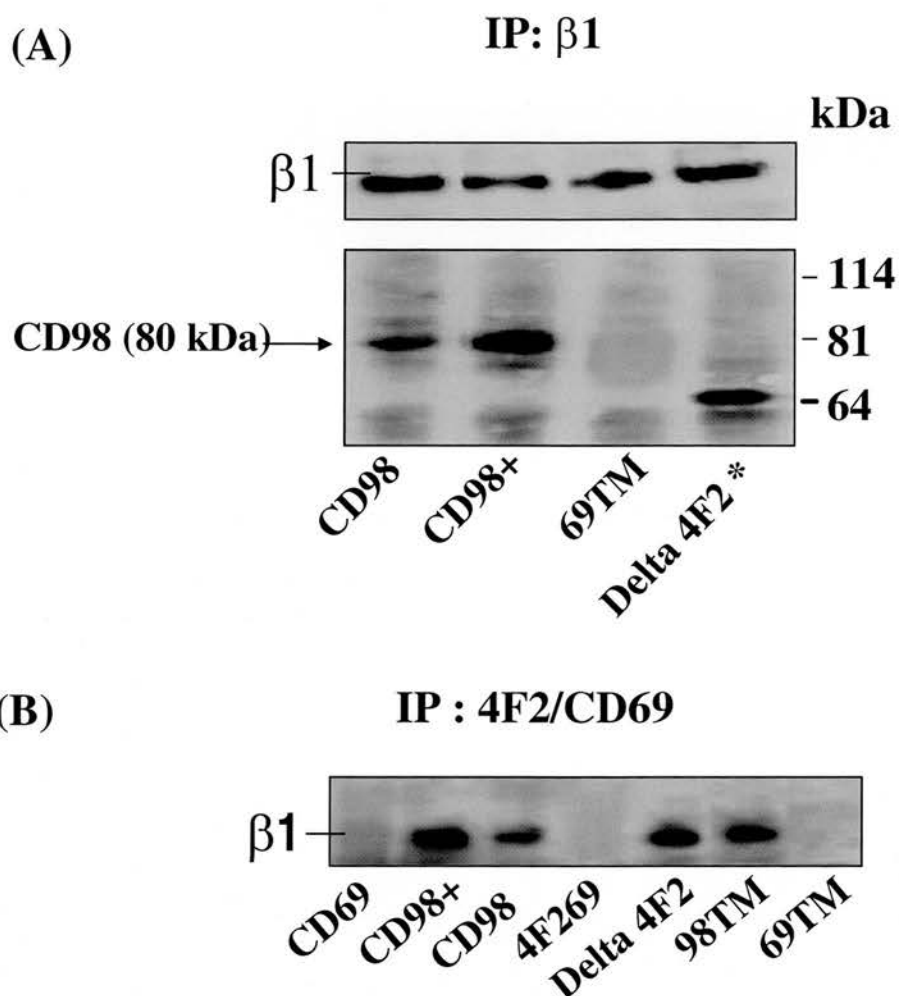


Figure 5.4.1 Association of $\beta 1$ integrin with CD98.

Cell lysates from CHOK1 cells stably transfected with CD98/CD69 and the chimeras were subjected to immunoprecipitation with antibodies against (A) $\beta 1$ or (B) 4F2 or CD69. Immunoprecipitates were resolved by SDS-PAGE and probed with antibodies against (A) CD98 and (B) $\beta 1$. * Delta 4F2 is a truncated CD98 mutant.

5.4.2 CD98 association with β 1 integrin in SCLC cells.

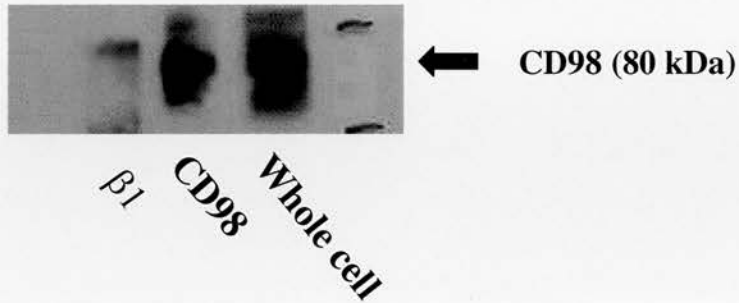
Figure 5.4.2a confirms that an association between β 1 and CD98 exists regardless of cell type. Immunoprecipitation was carried out on the SCLC cell line H69. β 1 integrin was immunoprecipitated using 2 μ g K20 and CD98 was immunoprecipitated using 2 μ g 4F2. Immunoprecipitations were resolved by SDS-PAGE and probed with antibodies against CD98 (goat polyclonal antibody SC-7095) and β 1 (mouse monoclonal antibody anti- β 1). In both panels (A) and (B), β 1 and CD98 co-immunoprecipitated with each other.

Dual label immunofluorescence was carried on SCLC cells, (Figure 5.4.2b). SCLC cells were incubated with antibodies against β 1 (9EG7) and CD98 (SC-7094). In all three cell lines H345, H69 and H510's, CD98 and β 1 localised together and this could be seen in the merged panels where areas of colocalisation appeared yellow. Panel (A) shows that the colocalisation seen between CD98 and β 1 was specific as no colocalisation was seen when cells were incubated with CD71, the transferrin receptor and CD98.

These experiments further confirmed my findings from the colocalisation studies that CD98 associates with β 1 integrin and that the transmembrane domain of CD98 is important for this association.

IP:β1/CD98

(A)



IP:β1/CD98

(B)

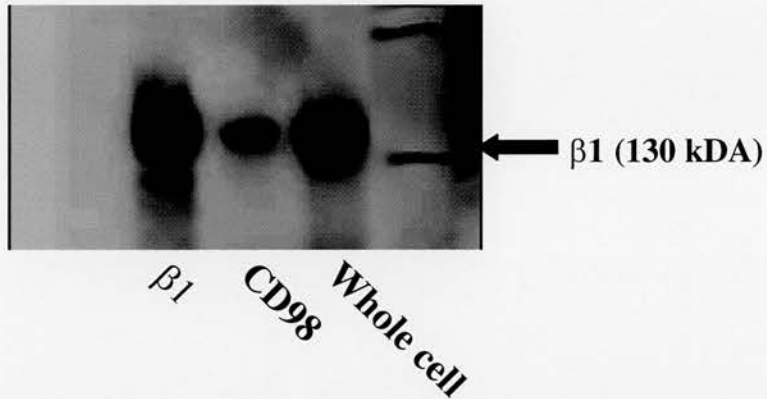


Figure 5.4.2a Association of $\beta 1$ integrin with CD98 in the SCLC cell line H69.

SCLC cell line H69 cell lysates were subjected to immunoprecipitation with antibodies against $\beta 1$ (K20) or CD98 (4F2). Immunoprecipitations were resolved by SDS-PAGE and probed with antibodies against (A) CD98 or (B) $\beta 1$. This immunoprecipitation confirms an association between $\beta 1$ and CD98 exists.

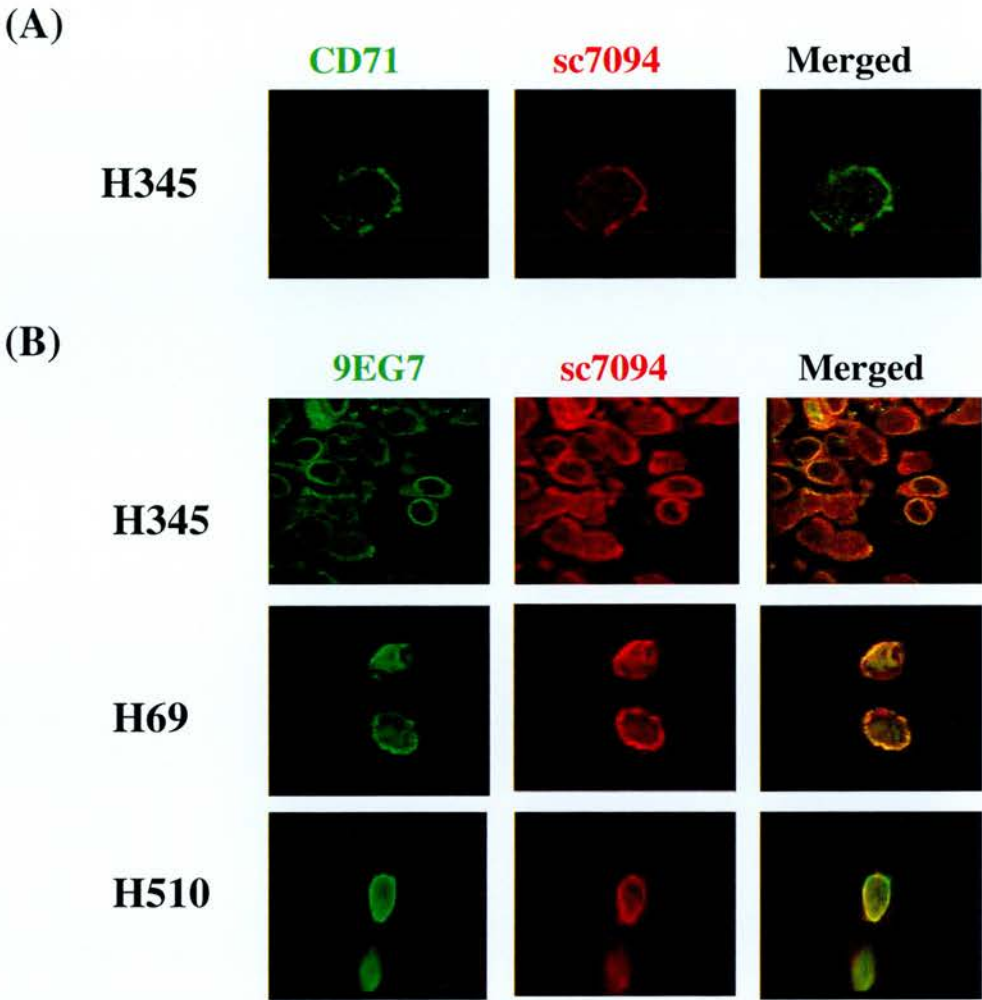


Figure 5.4.2b CD98 colocalises with β 1 integrin in the SCLC cell lines H345, H69 and H510's.

SCLC cells were incubated with antibodies against β 1 and CD98 as described in MATERIALS and METHODS. Confocal microscopy was performed using a TCS NT Confocal microscope system (Leica). (A) Localisation of the transferring receptor CD71 and CD98. (B) Localisation of β 1 (9EG7) and CD98 (sc7094) in the three SCLC cell lines. Areas of colocalisation can be seen in the merged panels and appear yellow. Images are of a single confocal plane and are representative of three independent experiments. CD98 and β 1 colocalise in all 3 SCLC cell lines. The negative control CD71 does not localise with CD98.

5.5 DISCUSSION

CD98 has recently been implicated as a regulator of integrin activation (Fencik et al., 1997). A genetic screen based on the physiological phenotype of integrin activation identified the Type II transmembrane glycoprotein CD98. CD98 could rescue integrins from Tac β 1- mediated integrin suppression. This indicated that CD98 played an important role in modulating integrin affinity.

Zent et al., (2000) found that CD98 associated with β_{1A} cytoplasmic domains using model protein mimics of dimerized integrin cytoplasmic tails. The specificity of this interaction was confirmed by lack of binding to mimics containing cytoplasmic domains from α_{IIb} or several other subunits indicating that CD98 interacted differentially with the β 1 tail in a class and splice variant specific manner. This finding also correlated with CD98's ability to complement dominant suppression. In this investigation CD98 was added to the β 1 tails in the presence of other cellular proteins so it remains possible that an intermediary protein is required for this interaction. A number of groups have been unable to crosslink the CD98 heavy chain to β 1 integrins, implying that the interaction between CD98 heavy chain and β 1 may not be direct (Kolesnikova et al., 2001). However the functional relevance of the β 1/CD98 complex is becoming more evident. Cho et al., (2001) revealed that there is a close link between CD98 function and β 1 by analyzing their respective aggregation properties. They found that although the aggregation produced by antibodies to both proteins shared many properties, there were also significant differences between the two pathways. EDTA inhibited β 1-mediated aggregation whereas CD98 induced aggregation is EDTA insensitive and therefore does not require divalent cations. They suggested that CD29 (β 1) plays a role in the inductive phase of the response rather than mediating the actual cell-cell adhesion. Warren et al., (2000) have also shown evidence of a functional association between CD98 and integrins in lymphocytes. Kolesnikova, et al., (2001), demonstrated that CD98 constitutively and specifically associates with β 1 intact heterodimers.

A further investigation into these findings and those of Rintoul et al., (2002), who demonstrated that CD98 and $\beta 1$ colocalised and co-immunoprecipitated in SCLC cells irrespective of activation state, could elucidate the physical association between these two molecules.

Using adherent CHOK1 cells stably transfected with either CD98, CD69 or the various CD98/CD69 chimeras, the association between CD98 and $\beta 1$ was investigated by dual label immunofluorescence and co-immunoprecipitation.

In order to carry out immunofluorescence, however, the staining techniques needed to be optimized. The main problem was in the form of the $\beta 1$ antibody and its effectiveness in binding to hamster $\beta 1$ integrin (CHO cells). After trying eight different $\beta 1$ antibodies, 9EG7 a rat anti-mouse monoclonal antibody was identified as the antibody which gave optimal staining. 9EG7 appeared to label in a punctuate fashion and was located mainly on the basal surface of the cells. This localization of $\beta 1$ has previously been described by Cook et al., (2002). They found that CD9 a tetraspanin (TM4SF) colocalised with $\beta 1$ integrins. They revealed that their integrin heterodimer $\alpha_5\beta_1$ of interest was located in punctuate patches across the basal surface and along filipodia. This filipodia staining of $\beta 1$ was seen when the chimeras were studied for colocalisation, an example of this can be seen when 98TM was analysed.

Rintoul et al., (2002) described the association between CD98 and $\beta 1$ occurred irrespective of the activation state of the integrin. Activating $\beta 1$, using a $\beta 1$ stimulating antibody TS216, had no effect on colocalisation and no effect was seen when a $\beta 1$ blocking antibody was used.

Interestingly when cells were incubated with 9EG7 at room temperature as described in MATERIALS AND METHODS, the $\beta 1$ was located within the perinuclear region and not the plasma membrane as expected. However when the cells were incubated with 9EG7 at 4°C overnight, the $\beta 1$ was localized within the plasma membrane.

Very little is known about the life cycle of $\beta 1$. However ECM-interacting integrins such as $\alpha_6\beta_4$, $\beta 1$, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ have been found by immunofluorescence to

reside in an intracellular vesicular compartment which at present has not been characterized (Miranti et al., 1998). Ng et al.,(1999) previously described the constant recycling of $\beta 1$. They found that in breast carcinoma cells activated $\beta 1$ integrin was located predominantly in the perinuclear/pericentriolar compartments distributed throughout the cytoplasm. Treatment with the PKC activator TPA caused redistribution of $\beta 1$ to the plasma membrane and a distinct perinuclear compartment. Incubating the cells at a lower temperature such as 20°C for 30 mins also lead to a dramatic redistribution of activated $\beta 1$ to the plasma membrane. They hypothesized that this dramatic redistribution of activated $\beta 1$ to the plasma membrane at 20°C implied that “there is continuous traffic of integrin to the cell surface, which is at least partially resistant to low temperature as well as a temperature –sensitive internalization process.

This finding appears to correlate with my observation that when the CHO cells were incubated with 9EG7 (an activating antibody) at 4°C, the $\beta 1$ was located in the plasma membrane. This may be due to the effect of low temperatures on the process which traffics integrins.

In addition, I found that fixation techniques also altered the location of $\beta 1$.

100% methanol and the mix of 50:50, methanol:acetone, caused excessive permeabilization and made the cells very leaky which effected the architecture of the cells. However using 3% PFA, allowed free access of the antigen but retained the subcellular architecture of the cells.

Having optimized the staining techniques, colocalisation between CD98 and $\beta 1$ was investigated. Using the chimeras, the component of the CD98 molecule which is necessary for colocalisation was determined. As shown by many groups, Zent et al.,(2000), Merlin et al., (2001) and Rintoul et al., (2002), CD98 and $\beta 1$ physically associate. My findings corroborate these observations. Using the CHOK1 cells stably transfected with CD98, CD69 or the chimeras, I have shown colocalisation between CD98 and $\beta 1$ in the CD98 stables and not in the negative control CD69 stables. The most striking find was that the colocalisation of CD98 and $\beta 1$ appeared to be dependent on the presence of the transmembrane domain of CD98. This observation supports my

findings from the previous chapter in which the transmembrane domain of the CD98 heavy chain played an important role in signaling and transformation. The chimera, 4F269 which had the transmembrane domain of CD98 missing and replaced with that of CD69 did not show colocalisation with $\beta 1$.

Deconvolution, an improved method of colocalisation analysis confirmed that the colocalisation seen between CD98 and $\beta 1$ existed and was not a consequence of non-specific labeling or poor resolution of the z- axis.

The physical association between CD98 and $\beta 1$ was further confirmed by co-immunoprecipitation experiments in both SCLC and CHOK1 cells stably expressing CD98. This result confirms the findings by Merlin et al., (2001) who showed that CD98 selectively co-immunoprecipitates with both LAT-2 and the $\beta 1$ integrin in epithelial cell lines. The transmembrane domain of CD98 was also found to be necessary and sufficient for this association. Co-immunoprecipitation of CD98 or $\beta 1$ was not seen in the chimera 4F269 which had the transmembrane domain of CD98 omitted. The immunoprecipitation experiments carried out on CHOK1 cells stably transfected with CD98, CD69 and the CD98/CD69 chimeras are very difficult to interpret. The cells were lysed and washed with a low stringency detergent. This detergent causes perforations to the cell membrane, breaking up the membrane into small pieces, but not completely solubilising it. Due to the less stringent nature of the detergent, the molecules may therefore be in the same place but may not necessarily be binding to each other. The other discrepancy in these results is a consequence of the chimeras having different extracellular domains and therefore being immunoprecipitated using different antibodies. 4F2 is a very good immunoprecipitating antibody compared to the anti-CD69 antibody. In this respect the immunofluorescence results are more viable than the immunoprecipitation results. It appears that the extracellular domain of CD98 which is much bigger in molecular weight to that of CD69 may play a role in the colocalisation of CD98 and $\beta 1$. The 98EX chimera which has the transmembrane and cytoplasmic domain of CD69 and the extracellular domain of CD98 colocalised with approximately 40% of $\beta 1$ integrins and co-immunoprecipitated with $\beta 1$ (results not shown). However

this chimera was very difficult to use, and despite double checking that this chimera was expressing the correct amino acid sequence; using sequence analysis, it still produced variable results in these experiments. It is therefore difficult to determine what role the extracellular domain of CD98 plays in integrin regulation without further experiments. Conversely it also appears that the transmembrane domain of CD98 is sufficient for colocalisation regardless of whether it consists of a CD98 or CD69 extracellular domain. The chimera 69TM failed to co-immunoprecipitate with $\beta 1$ and showed minimal colocalisation.

The D5 mutant, which lacks the amino acids 1-86, did not show colocalisation with the $\beta 1$ integrin. The amino acids 82-86 of CD98 were found to be critical for PI3-Kinase activation and anchorage independent growth in my last chapter. These results indicate that these amino acids 82-86 are also important for integrin association. However the mutant D5, also has the complete extracellular domain of CD98, but does not show colocalisation with $\beta 1$. This may be a consequence of these four amino acids being absent. With these amino acids missing, it may cause a conformational change in the CD98 heavy chain which is unfavourable for integrin association and signalling. FACS data shows that both 98EX and D5 are expressed on the cell surface. This could therefore imply that the absence of 82-86 amino acids changes the way CD98 orientates in the membrane, preventing the association of $\beta 1$ and CD98. This hypothesis may also explain why 69TM did not colocalise. 69TM consists of the extracellular and cytoplasmic domain of CD98 but has the transmembrane domain of CD69. This transmembrane domain of CD69 may also cause a conformational change to the CD98 extracellular domain due to a possible difference in the amino acids sequence in the transmembrane domain compared to that of CD98. This dependency on the transmembrane domain for integrin regulation has also been described by Fencik et al., (2001). They found using the same chimeras which were kindly donated to us, that the cytoplasmic and transmembrane domains of CD98 were required for interactions with integrin cytoplasmic domains, specifically its ability to complement dominant suppression. They hypothesized that the CD98 transmembrane domain may influence the conformation of the cytoplasmic domain to promote binding to integrin cytoplasmic

domains. However this present study has investigated the full length $\beta 1$ molecule rather than just the cytoplasmic tails and the results seem to indicate that the $\beta 1$ molecule may bind to either the extracellular domain of CD98 or that the transmembrane domain may induce a change in the conformation of the extracellular domain promoting CD98 and $\beta 1$ association.

The biochemical mechanisms by which CD98 and integrins functionally interact are still unclear. There is evidence that intermediary proteins may be involved in the association of CD98 and $\beta 1$. Zent, et al., (2000) found that CD98 associated with $\beta 1A$ cytoplasmic domains but this experiment was carried out in the presence of other cellular proteins such as talin and filamin. Kolesnikova, et al.,(2001) found that GPI-linked proteins such as CD109, TM4SF proteins such as CD81 and CD147 which are found in substantial levels in the light membrane fraction, did not co-immunoprecipitate with CD98. This lead to their hypothesis, that the association of CD98 with integrins has another level of specificity beyond simply co-localizing in light membrane fractions. This implies that the interaction between CD98 heavy chain and $\beta 1$ may be direct.

The heavy chain of CD98 contains two extracellular cysteines. Kolesnikova, et al., (2001), mutated these cysteines to serine. The C330S mutant retained association with endogenous murine $\beta 1$ in NIH 3T3 cells, but the C109S mutant showed no detectable $\beta 1$ association. The association of the CD98 heavy chain with the CD98 light chain was maintained for the C330S but lost with the C109S mutant. The mutation of cysteine 109 disrupted the disulphide linkage to the light chain. These results suggested that the CD98 light chain may be necessary for the CD98-integrin association. Mutation of these cysteines however, which was found to disrupt the CD98 heavy chain, light chain association and reduce amino acid transport, did not disrupt its *in vitro* binding to β_{1A} integrins and its effects on integrin activation, (Fenczik, et al., 2001). These results indicate that the covalent association of the CD98 heavy chain with a light chain is not required for its interaction with integrins.

These results seem to confirm an association between CD98 and $\beta 1$ exists. Immunoprecipitation is the most widely used immunochemical technique to study

protein interactions. It also enables the detection of rare proteins which would otherwise be difficult to detect, as proteins can be concentrated up to 10,000 fold. Immunofluorescence however allows visualization of small molecules, and interactions between molecules can be seen in both fixed and live cells. However in order to investigate the interaction between CD98 and $\beta 1$ more thoroughly, Fluorescence Resonance Energy Transfer (FRET) could be used. FRET detects the proximity of fluorescently labeled molecules over distances $> 100 \text{ \AA}$. It can be used to map protein-protein interactions *in vivo*. It can also be used in a confocal capacity to analyze colocalisation (Kenworthy, 2001).

Many groups have indicated a functional association between CD98 and $\beta 1$. CD98 has been shown to play an important role in many cellular processes such as adhesion, fusion, migration and metastasis in which integrin regulation is important in modulating these processes. It then seems plausible that a physical association between CD98 and $\beta 1$ exists.

The data presented here looking at the whole $\beta 1$ integrin molecule rather than the cytoplasmic tails indicates that this physical association between CD98 and $\beta 1$ exists and that as reported the extracellular domain and the amino acids 82-86 of the transmembrane domain of the CD98 molecule are important for this interaction.

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CHAPTER 6

CD98 AND ITS RELATIONSHIP WITH NCAM

6.0 INTRODUCTION

Many papers have reported that CD98 associates with a variety of different molecules. Rintoul et al., (2002), showed that CD98 and $\beta 1$ colocalised in SCLC and I have now shown that $\beta 1$ and CD98 physically associate using co-immunoprecipitation experiments. Suga et al., (2001) reported that CD98 stimulation by anti-CD98 monoclonal antibodies induced the activation of $\beta 2$ -integrin (LFA) via the PI3-Kinase pathway, activating Rap-1. Kakugwa et al., (2003), showed that CD98 co-immunoprecipitated with CEA-CAM and stimulated CEA-CAM-1 mediated cell adhesion. These findings suggest that CD98 acts as a “molecular facilitator” (Rintoul et al., 2002), in the plasma membrane by regulating different types of adhesion molecules. Having looked at the diverse effects of CD98 it seemed plausible to investigate whether there are any other molecules like CD98 which have similar effects on integrins.

NCAM, Neural cell adhesion molecule is a membrane associated glycoprotein of the Ig superfamily. It plays an important role in neural development and neural regeneration. Three isoforms of NCAM are generated through alternative splicing; 2 transmembrane isoforms NCAM-140 and NCAM-180 and a 120kDa glycosylphosphatidylinositol-linked (GPI) isoform, (Figure 6). L1 is another member of the Ig superfamily which plays a similar role in developmental processes as NCAM. It is a 200 kDa glycoprotein which is highly expressed on neuronal cells. Both NCAM and L1 have been shown to engage in homophilic binding (cell-cell) interactions. However more recently, they have been shown to also exert heterophilic cis and trans binding interactions with molecules of different classes, such as the cell adhesion molecule, members of the FGFR family and extracellular components (Seidenfaden et al., 2003). NCAM has been shown to play an important role in the progression to tumour

malignancy. For example, in various cancer types, the expression of NCAM shifts from the adult 120 kDa isoform to the embryonic 140 and 180 kDa isoforms, together with a general downregulation of expression (Cavallaro and Christofori, 2001; Christofori, 2003). Perl et al., (1999), reported that abrogation of NCAM expression in pancreatic β cell tumours results in a dramatic increase in the incidence of metastases. NCAM-deficient tumour cells were shown to have an impaired cell-substrate adhesion, whilst cell-cell adhesion was not affected by lack of NCAM, (Cavallaro et al., 2001). This indicates a possible role for CAMS and specifically NCAM in modulating cellular functions, critical for tumour progression (Christofori, 2003).

Reports of mechanisms involving direct L1-integrin ligation and the induction of transient integrin signaling and endocytosis. have recently been documented (Mechtersheimer et al., 2001; Thelen et al., 2002). Silletti et al., (2004), have shown that L1 promotes the expression and utilization of integrin $\alpha_5\beta_3$ and speculated that this discovery has broad mechanistic ramifications for the participation of L1 in a variety of normal and pathological processes, such as neurite extension, extravasation, myelination, and metastasis. Cavallaro et al., (2001), showed that NCAM binds to and stimulates FGFR4 stimulated tyrosine kinase activation of pancreatic tumour cells. They hypothesised that modulation of tumour cell-matrix adhesion by NCAM, affects integrin regulation. This may occur by NCAM inducing the formation of a signalling complex; activating signal transduction pathways, similar to the induction of integrin signalling by L1.

These hypotheses lead to the investigation of whether CD98 and NCAM are similar molecules in the terms of modulating integrin signalling and whether CD98 and β_1 could form a signalling complex.

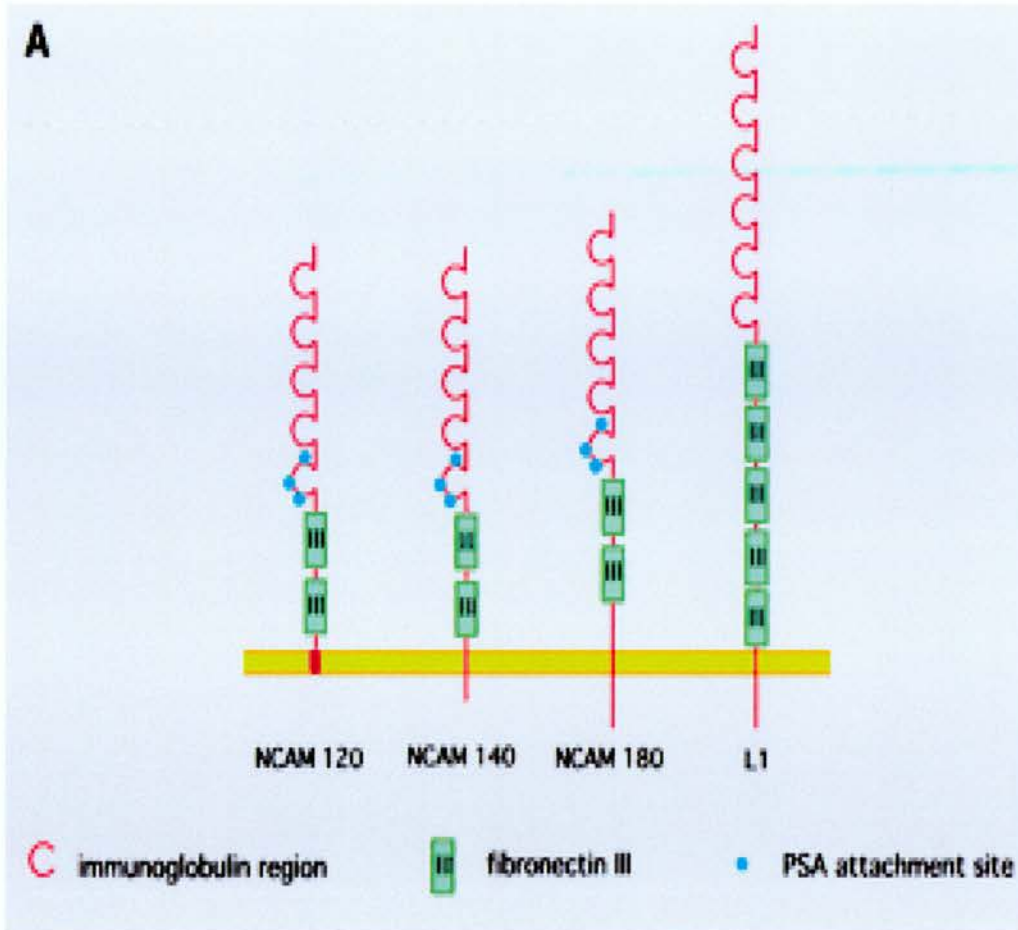


Figure 6 Structure of neural cell adhesion molecule (NCAM) and L1.

The extracellular part of all NCAM isoforms consists of 5 immunoglobulin-like and 2 fibronectin type III-like domains, whereas that of L1 contains 6 immunoglobulin-like and 5 fibronectin type III-like domains. The 120 kDa NCAM isoform is linked to the membrane through a phospholipid anchor and thus can be easily released into the extracellular milieu. The 140 kDa NCAM, 180 kDa NCAM, and L1, in contrast, are anchored to the cell membrane and have specific intracellular domains, through which they can interact with the cytoskeleton or components of the postsynaptic density. Blue dots demarcate the attachment sites for polysialic acid (PSA) polymers on NCAM (Welzl and Stork, 2003).

6.1 CD98 and its relationship with NCAM.

6.1.1. *Expression of NCAM isoforms in SCLC cell lines.*

From the evidence gathered thus far, it is becoming evident that CD98 is an important molecule in regulating integrin signalling. It now seems plausible that CD98 may be part of signalling complex with $\beta 1$ which modulates various signals. Fencik et al., (1997) found that antibody mediated crosslinking of CD98 stimulated $\beta 1$ integrin-dependent cell adhesion. Suga et al., (2001) demonstrated that CD98 stimulation by anti-CD98 mAbs induced activation of $\beta 2$ -integrins (LFA-1) via PI3-Kinase and Rap-1 activation. Kakugawa et al., (2003), reported that CD98 stimulation could activate CEA-CAM-1 mediated cell adhesion via protein kinase C δ (PKC δ) activation. These results indicate that CD98 can regulate different types of adhesion molecules. Therefore this hypothesis was tested and it was determined whether there were any other molecules like CD98, which could have similar effects on integrin activation. NCAM, Neural Cell Adhesion Molecule, is a membrane associated glycoprotein of the Ig superfamily and plays an important role in neural development and neural regeneration. It is encoded by a single gene, but a number of different isoforms are generated through alternative RNA splicing, eg. NCAM-120, NCAM-140 and NCAM-180. NCAM engages in homophillic (NCAM-NCAM) and heterophillic binding (NCAM-ECM etc) (Ditlevsen et al., 2003). Homophillic binding of NCAM has been shown to lead to the activation of a number of intracellular signalling pathways. An example of this is induction of neurite outgrowth through pathways activating FGFR, PKC and MAPK (reviewed by Berezin et al., 2000). Cavallaro et al., (2001), hypothesised that NCAM assembles a FGFR signalling complex leading to $\beta 1$ integrin mediated neurite outgrowth and matrix adhesion. These findings lead to my hypothesis that CD98 associates with NCAM, $\beta 1$ and FGFR forming a complex which can modulate signalling.

SCLC, small cell lung cancer, is a highly malignant tumour, comprising of cells which have endocrine characteristics and in culture grow as floating spheroid aggregates. However NSCLC, non-small cell lung cancer, cells grow as adherent

monolayers (Pettinghill et al., 1980). The reason for the molecular differences between SCLC and NSCLC has not yet been established. However one potential candidate for these changes in adhesive properties of these cells is NCAM.

To confirm that endogenous NCAM was present in SCLC cells, quiesced cells were lysed as described in MATERIALS AND METHODS and then run on SDS-PAGE and western blotting was carried out using a mouse monoclonal antibody raised against human NCAM. The blots were then probed with a secondary mouse HRP and visualized using enhanced chemiluminescence (Figure 6.1.1). From three independent experiments it was found that NCAM appears to exist in different isoforms depending on the SCLC cell line. H345 only showed NCAM-180 whereas H69 showed both NCAM-180 and NCAM-140. Interestingly in many cancers, the expression of NCAM changes from the isoform NCAM-120 that is linked to GPI (glycophosphatidylinositol) to the transmembrane isoforms NCAM-140 and NCAM-180 in the progression to tumour malignancy.

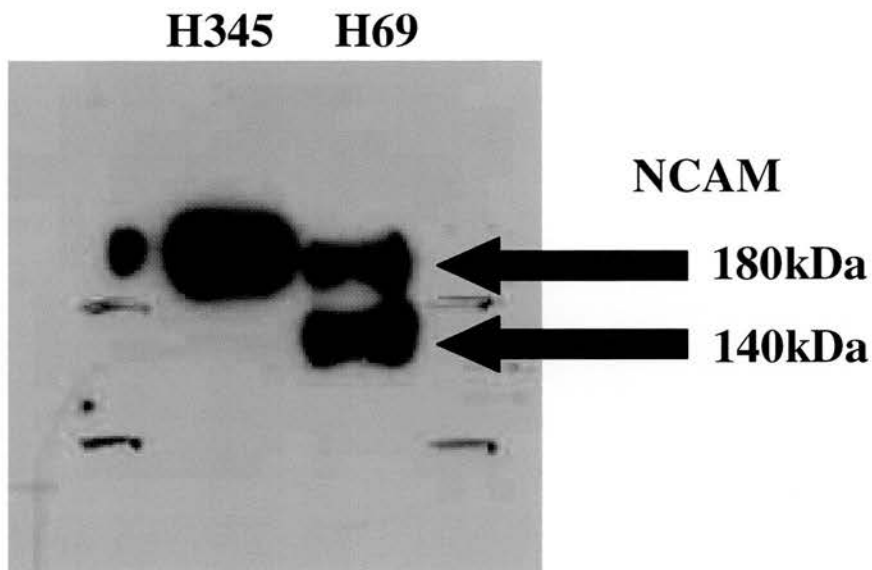


Figure 6.1.1 Western blot of NCAM expression in two SCLC cell lines H345 and H69.

Quiesced SCLC cells were lysed and their protein concentration was determined as described in MATERIALS AND METHODS. Aliquots of lysate were then run on SDS-PAGE and western blotted with an anti-NCAM antibody. The blots were then visualized using enhanced chemiluminescence. This blot confirmed that endogenous NCAM was present in SCLC cells. NCAM appeared to exist in different isoforms depending on the SCLC cell line.

6.1.2. NCAM colocalises with CD98.

Having established that endogenous NCAM exists in different isoforms in different SCLC cell lines and that there is a high level of expression of CD98 in SCLC, an investigation into whether these two molecules associated with each other was carried out. This was investigated using dual label immunofluorescence.

SCLC cells were adhered to coverslips using poly-L-lysine and left to grow and adhere overnight to the matrix at 37°C. Cells from both H69 and H345 cell lines were incubated with antibodies against NCAM and CD98 and then the corresponding secondary alexa fluors as described in MATERIALS AND METHODS. Images were visualized using confocal microscopy (Figure 6.1.2a). Images are representative of four independent experiments. Labelling of NCAM and CD98 (sc7094), appeared to be present around the cell membrane. When these images were merged, colocalisation of both these molecules was visible as the cell membranes were yellow in colour. This phenomenon occurred in both cell lines H69 and H345. This colocalisation was confirmed by analysis with Bitplane Imaris 4 software and the % of total colocalisation was calculated. 65% colocalisation was seen between CD98 and NCAM as shown in figure 6.1.5b and 6.1.5c.

In order to confirm this colocalisation seen was real and not a consequence of non-specific binding, adhered SCLC cells were incubated with negative control antibodies, Ig2a (isotype control) and the secondary alexa red 568 (goat-anti-mouse, Figure 6.1.2b). No specific labelling was seen in the top panels, with either of these controls. However to confirm this further, the membrane bound transferrin receptor CD71 was tested in colocalisation studies with NCAM (Figure 6.1.2b). When images of CD71 labelling and NCAM labelling were merged, no colocalisation was seen, the labelling of each molecule appeared to be quite separate. Colocalisation analysis assured that this finding was correct as only 14 % of NCAM colocalised with CD71 as shown in figure 6.1.5c.

This confirmed that the colocalisation seen between CD98 and NCAM was not a consequence of non-specific binding.

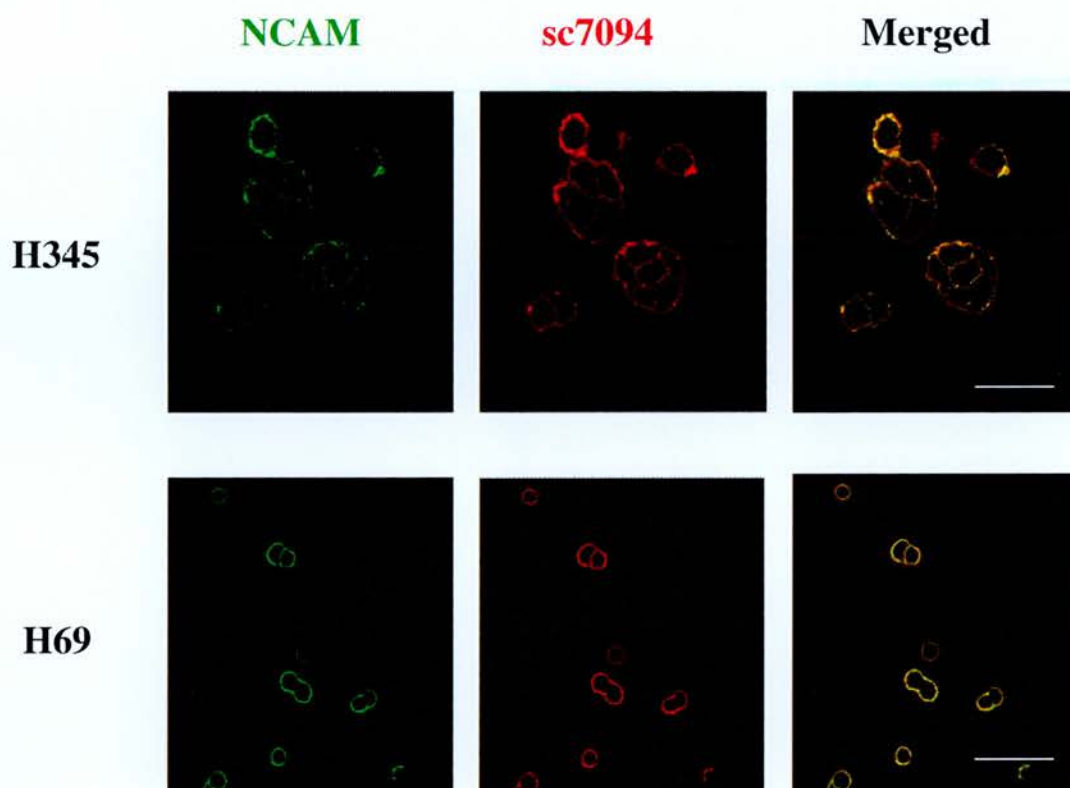


Figure 6.1.2a NCAM and CD98 colocalise in the SCLC cell lines H345 and H69.

SCLC cells were incubated with antibodies against NCAM and CD98 as described in MATERIALS AND METHODS. White bars, 4.4 μ m and 8.8 μ m respectively. Confocal microscopy was performed using a TCS NT Confocal microscope system (Leica). Localisation of NCAM (green) and CD98 (sc7094, red) are shown in the top and bottom panels. Areas of colocalisation can be seen in the merged panels and appear yellow. Images are of a single confocal plane and are representative of 3 independent experiments. CD98 and NCAM colocalise in both H345 and H69 SCLC cell lines.

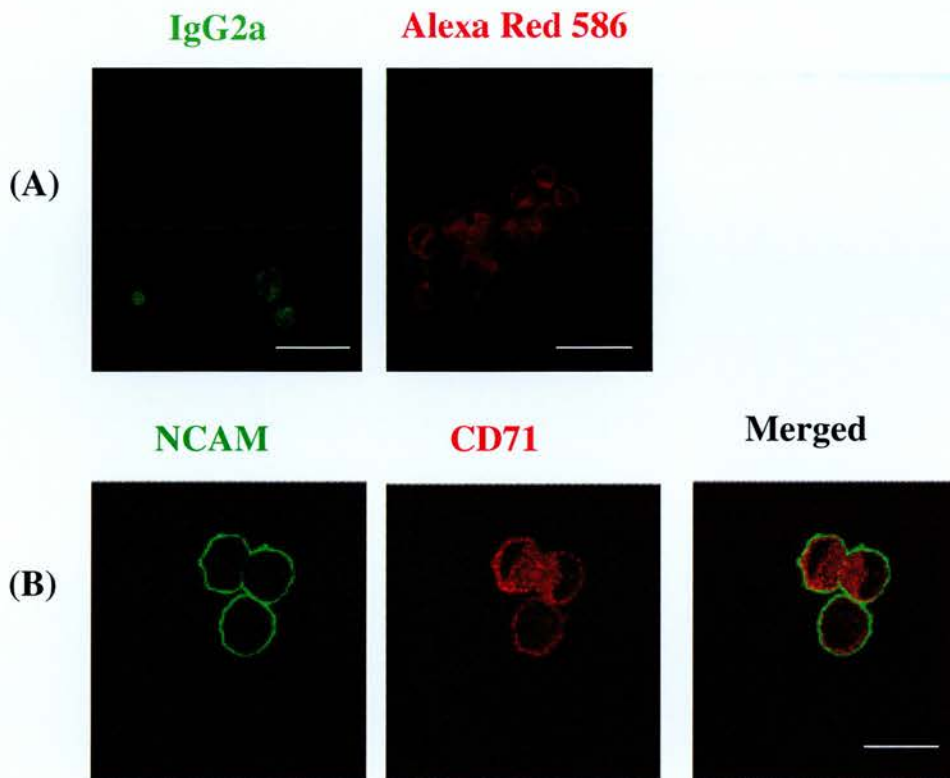


Figure 6.1.2b CD71 does not colocalise with CD98 in the SCLC cell line H69.

SCLC cells were incubated with antibodies as described in MATERIALS AND METHODS. Confocal microscopy was performed using a TCS NT Confocal microscope (Leica). (A) Testing negative control antibodies, isotype control IgG2a antibody and secondary alexa red antibody. White bars, 8.8 μ m. (B) Localisation of CD71 and NCAM. White bar, 3 μ m. No evidence of non specific binding was seen when cells were incubated with the negative control antibodies. The transferrin receptor CD71 does not colocalise with NCAM.

6.1.3. NCAM colocalises with $\beta 1$ integrins.

$\beta 1$ spectrin is a ubiquitous scaffolding protein with a functional unit made up of an α and β heterodimer. Leshchyn'ska et al., (2003), showed that NCAM-180, 140 and 120 colocalised with $\beta 1$ spectrin. Cavallaro et al., (2001), found when adherent NCAM^{+/+} tumour cells were treated with neutralizing antibodies against $\beta 1$, neurite outgrowth was abrogated. They also found that expression of the different NCAM isoforms by transient transfection in NCAM^{-/-} tumour cells, restored neurite outgrowth and cell adhesion, which in turn was repressed by antibodies against $\beta 1$. This evidence indicated a possible role for $\beta 1$ integrin in NCAM signalling.

Having established that CD98 and $\beta 1$ physically associate, (Chapter 5) and NCAM and CD98 colocalise, an investigation was carried out to see if $\beta 1$ and NCAM associate with each other, thereby forming a complex with CD98.

SCLC cells were adhered to coverslips, incubated with antibodies against NCAM and $\beta 1$ integrin and then their corresponding secondary antibodies. Confocal microscopy was performed and colocalisation between the two molecules was examined (figure 6.1.3). Labelling of NCAM was found around the cell membrane and as described earlier in Chapter 5, the $\beta 1$ was found around the cell membrane, but in a punctuate fashion. When these images were merged, partial colocalisation was observed around the membrane. This colocalisation was confirmed by using Bitplane Imaris 4 colocalisation software, 46% of NCAM colocalised with $\beta 1$, as shown in figure 6.1.5c. Images are representative of 2 independent experiments.

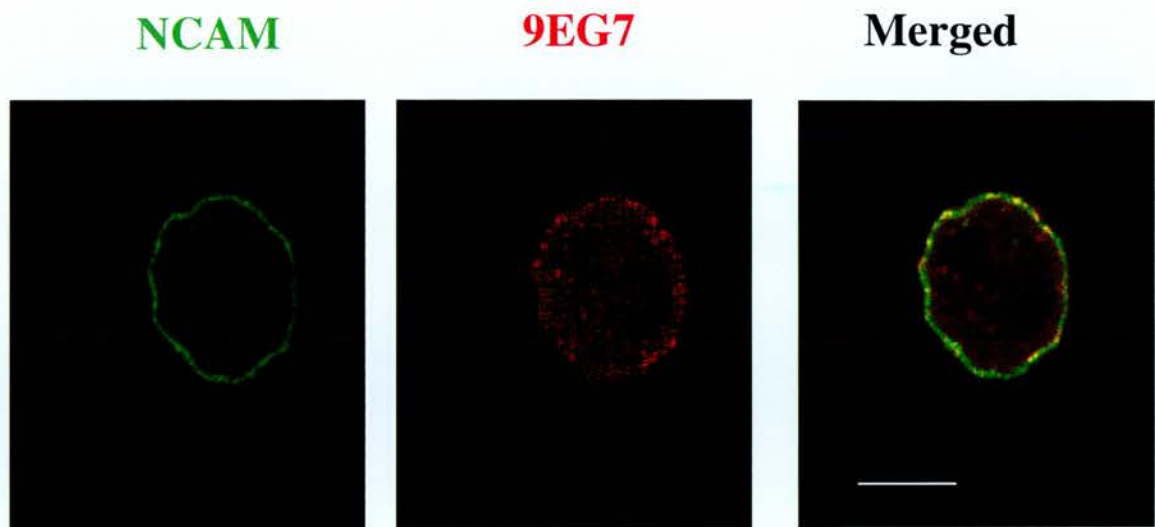


Figure 6.1.3 NCAM partially colocalises with $\beta 1$ integrin.

SCLC cells were incubated with antibodies against NCAM and $\beta 1$ as described in MATERIALS AND METHODS. White bar $5\mu\text{m}$. Confocal microscopy was performed using a TCS NT Confocal microscope system (Leica). Localisation of NCAM (green) and $\beta 1$ (9EG7, red) are shown in the 1st and 2nd panels. When these two images are merged, areas of colocalisation appear yellow. Images are of a single confocal plane and are representative of 2 independent experiments.

6.1.4. Co-immunoprecipitation of CD98, NCAM and β 1 integrin.

NCAM has been shown to associate with β 1 spectrin, FGFR and PKC. Beggs et al., (1997), found that NCAM-140 coimmunoprecipitated with p125^{fa}k and p59^{fy}n. These findings suggest that by forming a molecular complex, tyrosine kinases are activated initiating a signalling cascade. In order to confirm the association between CD98 and NCAM further and to look at the physical association of NCAM and β 1 in greater detail, co-immunoprecipitation experiments were performed.

Immunoprecipitation was carried out on the SCLC cell line H69. Protein lysates were made from the SCLC cells and β 1, CD98, NCAM or CD71 were immunoprecipitated using either, K20, 4F2, anti-NCAM and anti-CD71 (2 μ g) respectively. These immunoprecipitates were washed and resolved by SDS-PAGE and probed with antibodies against CD98, β 1 or NCAM. The blots shown below are representative of four independent experiments.

Figure 6.1.4 shows in the top panel that NCAM and β 1 associates with CD98 with corresponding bands at ~85 kDa. The middle panel shows that NCAM and CD98 associate with β 1 again with bands corresponding to β 1 at 130 kDa. The bottom panel confirms these observations as CD98, and β 1 co-immunoprecipitate with NCAM showing corresponding bands at mainly 140 kDa but also at 180 kDa. However the negative control, CD71 showed no immunoreactivity with NCAM or CD98. This colocalisation and association was then confirmed by analysis using the Bitplane Imaris 4 software, 66% of CD98 and 46% of β 1 colocalised with NCAM whereas the colocalisation seen between CD71 and NCAM was minimal at 14% as shown in Figure 6.1.5.

These experiments confirmed my colocalisation studies that CD98 and NCAM physically associate with each other. NCAM and β 1 appear to be located in the same place on the membrane as partial colocalisation was seen between the two molecules and also physically associate together as shown in the co-immunoprecipitation experiments. This experiment was also carried using the SCLC cell line H345 and the same results

were seen. However more experiments need to be carried out to obtain a more conclusive answer.

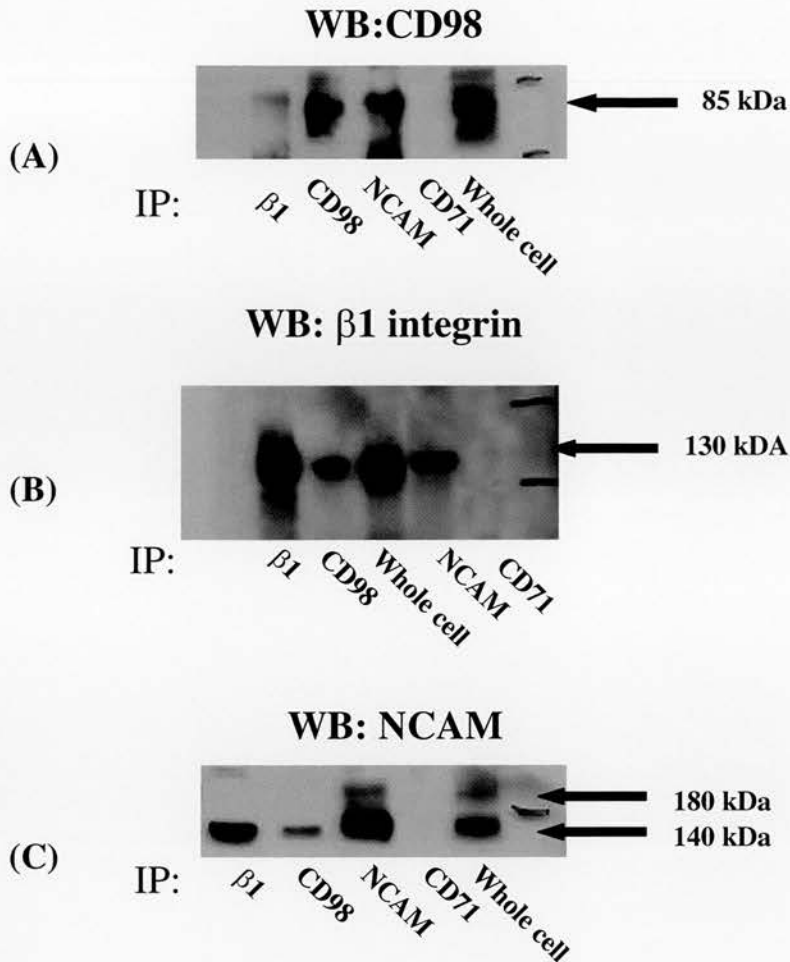


Figure 6.1.4a) Association of CD98 with β 1 and NCAM and association of NCAM with β 1.

SCLC cell line H69 cell lysates were subjected to immunoprecipitation with antibodies against β 1 (K20), CD98 (4F2), NCAM (anti-NCAM) and CD71 (anti-CD71). Immunoprecipitations were resolved by SDS-PAGE and probed with antibodies against (A) CD98, (B) β 1 and (C) NCAM. CD98 co-immunoprecipitated with both β 1 and NCAM. NCAM co-immunoprecipitated with CD98 and β 1. β 1 co-immunoprecipitated with NCAM and β 1. No association was seen between NCAM and CD71 or between CD98 and CD71.

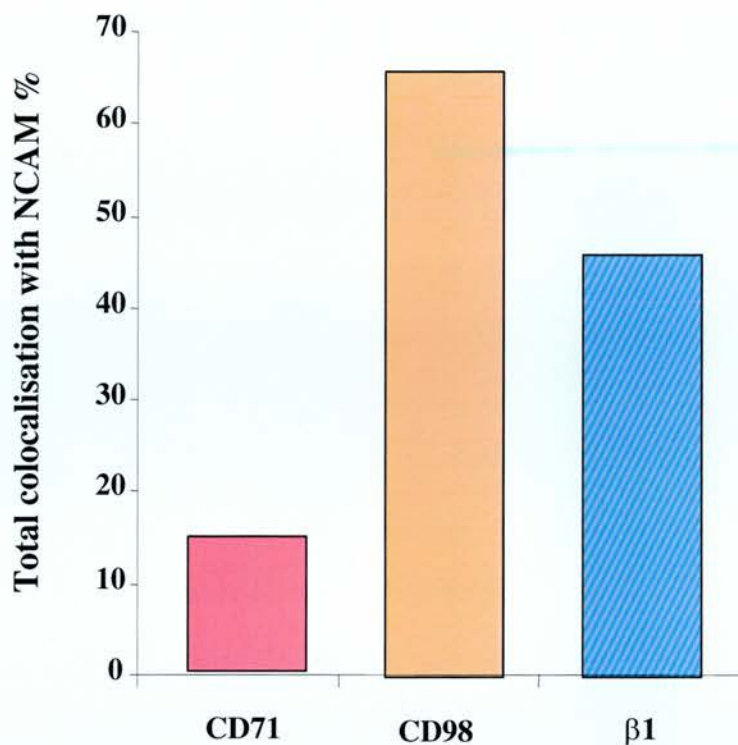


Figure 6.1.4b). Colocalisation of NCAM with various molecules.

Confocal images were analysed using Bitplane Imaris 4 software and the % of colocalisation of NCAM with CD98, β1 and CD71 was calculated. The results are expressed as % of colocalisation, (n=1).

The colocalisation seen between NCAM and CD71 was minimal at ~14% compared to the colocalisation between CD98 and β1; 66% and 46% respectively.

6.1.5. Functional Effects of NCAM

Having established that CD98 and NCAM colocalise and associate together, its functional relevance was investigated. Moolenaar et al., (1990) and Kibbelaar et al., (1989) recently demonstrated that a dominant surface antigen of SCLC cells is NCAM. NCAM may potentially be important in the early metastasis characteristic of SCLC cells. A potential candidate for the changes seen in NSCLC cells in terms of their adhesive properties is NCAM. Kibbelaar et al., (1989) found that 20% of patients with NSCLC whose tumours were positive for NCAM had a significantly shorter survival than did NSCLC patients with tumours negative for NCAM immunoreactivity.

Anchorage independence was measured using a colony assay based on the property of tumour cells to metastasise and grow in inappropriate locations *in vivo*. This technique is discussed in more detail in section 4.1.1. To investigate whether the expression of NCAM enhanced anchorage independence, colony assays were carried out using H69 SCLC cells.

NCAM-Fc, a NCAM fusion protein (kindly donated to us by Melitta Schachner, (Zentrum für Molekulare Neurobiologie, Universität Hamburg, Germany) was used to stimulate the endogenous NCAM present within the SCLC cells. Beggs et al., (1997) found that clustering of NCAM-140 at the neuronal cell surface either by antibodies or by treatment with a soluble NCAM fusion protein (NCAM-Fc) caused transient phosphorylation of both p59^{l^{yn}} and p125^{FAK}. Schmid et al., (1999) demonstrated that when NCAM-140 expressing B35 neuroblastoma cell were treated with NCAM Fc, the stimulation of phosphorylation of MAPK's increased four-fold.

NCAM-Fc was transfected into CHOK1 cells and the secreted fusion protein was collected and then purified from the medium by protein A-Sepharose chromatography as described in MATERIALS AND METHODS Section 2.17. SCLC cells were suspended in 0.3% agarose containing 2µg/ml of a NCAM-Fc. This suspension was layered on top of the 0.5% agarose in 1% FCS and the number of colonies was counted after 14 days using a light microscope as described in MATERIALS AND METHODS. The results, shown in figure 6.1.6, suggest that NCAM enhanced clonal growth of SCLC cells. After

7 days the cloning efficiency of NCAM stimulated cells was approximately 15% compared to the 10% shown by control SCLC cells. However this difference is further increased after 14 days where NCAM stimulated cells show a cloning efficiency of approximately 60% compared to the 40% shown by control cells. Cells stimulated with 4F2 showed an increased cloning efficiency compared to control cells but did not further increase growth in cells stimulated with either 1 or 2 $\mu\text{g/ml}$ NCAM-Fc. As well as increased cloning efficiency there was also a noticeable increase in size of the colonies in the presence of NCAM-Fc and 4F2.

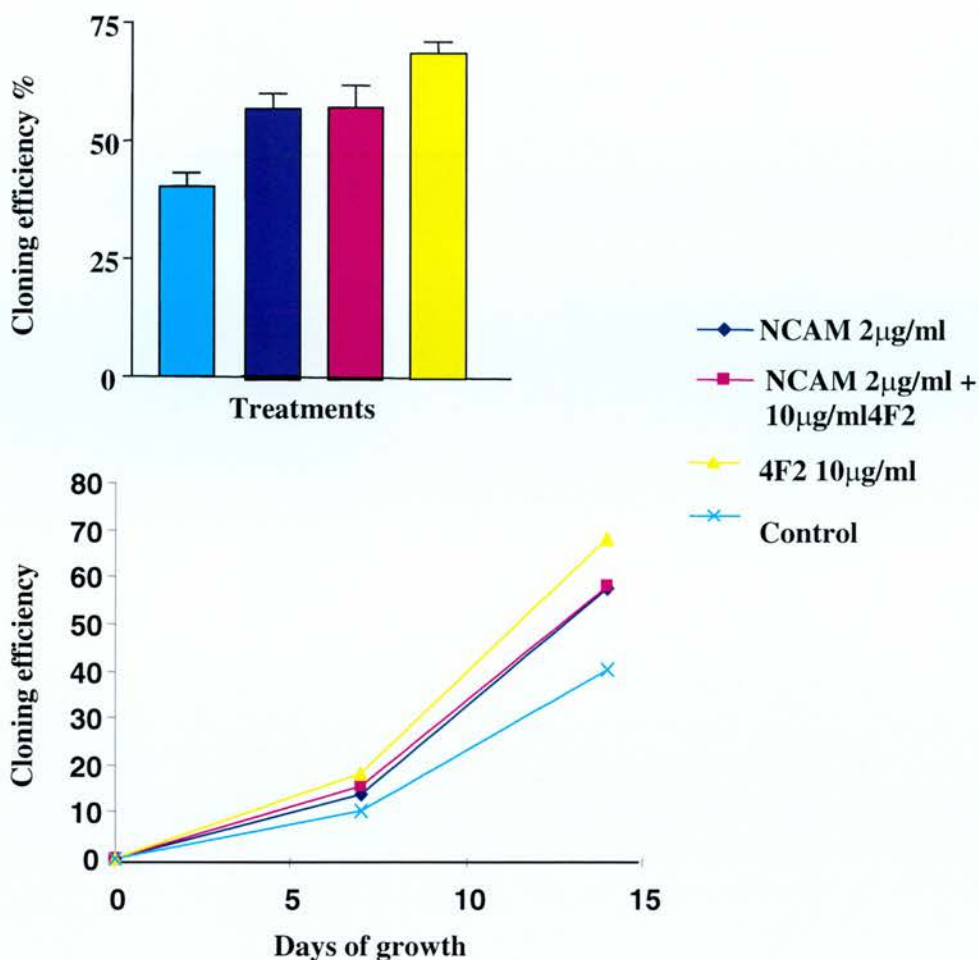


Figure 6.1.5. NCAM may cause an increase in clonal growth after 14 days compared to untreated H69 SCLC cells.

The clonal growth of H69 SCLC cells treated either with 1 or 2μg/ml NCAM-Fc or 10 μg/ml 4F2 in semi-solid 0.3% agarose medium containing 1% FCS was determined after 7 and 14 days as described in MATERIALS AND METHODS. When cells were treated with both 4F2 and NCAM-Fc there was no obvious difference in their cloning capabilities. Results are expressed as % cloning efficiency and are the mean of 2 independent experiments

6.1.6. SCLC cell adhesion to laminin is enhanced by either 4F2 or NCAM.

To investigate the effect of NCAM on integrin function cell attachment assays were carried out. 96 well tissue culture plates were coated with 10µg/ml laminin, or 100µg/ml poly-L-lysine as positive controls as described in MATERIALS AND METHODS, section 2.16. After blocking, 1×10^5 H69 SCLC cells were plated in the presence or absence of 10µg/ml 4F2, 2µg/ml NCAM-Fc or both and left at 37°C to incubate for 1hr. After washing and fixation with 3% PFA, methylene blue was added to the wells and the colour is developed by adding 0.1M HCl. The graph in figure 6.1.7 is the mean of 2 independent experiments and shows the percentage of adhesion to laminin etc, compared to poly-L-lysine (100%). Adhesion to laminin was enhanced in the presence of 4F2 or NCAM. There was approximately a 40% increase in adhesion to laminin when stimulated with 4F2 and approximately a 25% increase when stimulated with NCAM. Stimulation with both NCAM-Fc and 4F2 had no significant effect on adhesion to laminin. When the cells were plated onto NCAM-Fc, no adhesion occurred, (data not shown). These results suggest a possible role for NCAM in anchorage independent growth and cellular adhesion events but more experiments need to be carried out to obtain a more conclusive answer.

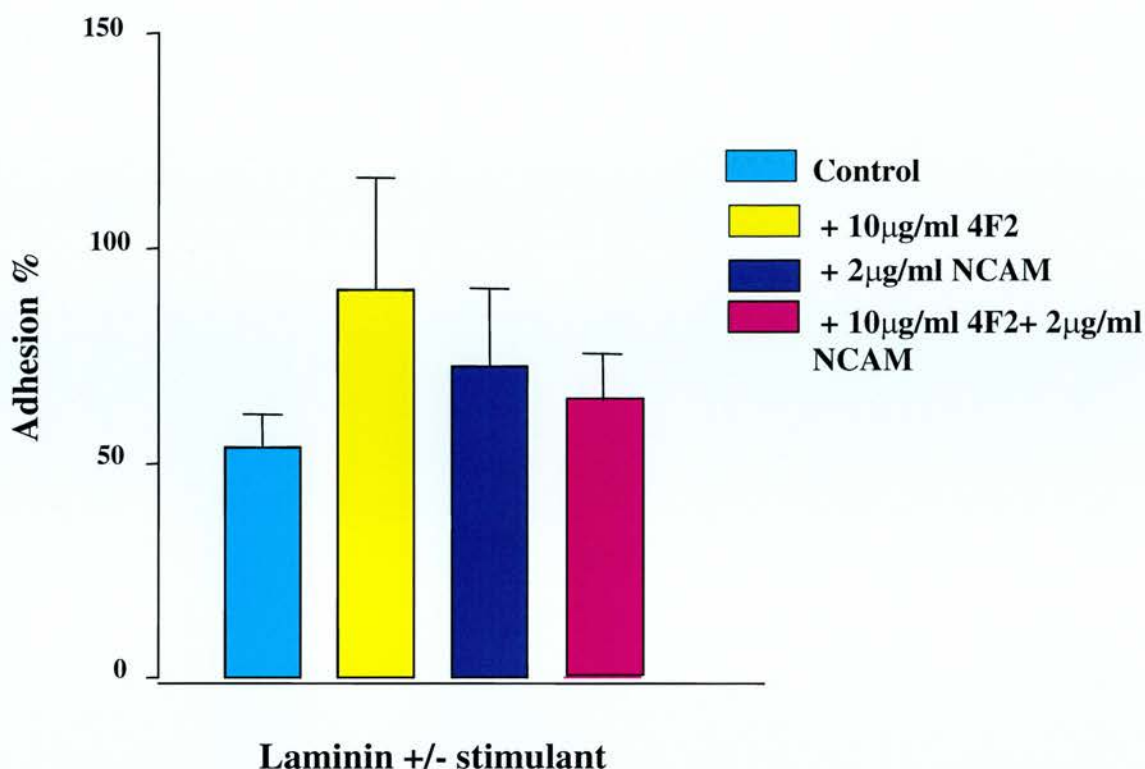


Figure 6.1.6. The effect of 4F2 and NCAM on the adhesion of the SCLC cell line H69 to the ECM.

A 96 well tissue culture plate was coated with 10mg/ml laminin for 1hour at 37°C, blocked with 2% BSA for 1hr. The cells were then plated in the presence of 2µg/ml NCAM-Fc or 10µg/ml 4F2 or both and incubated at 37°C for 1hr. Cell adhesion was then determined by staining the wells with methylene blue and elution with 0.1M HCl. The attachment of H69 cells to wells coated with Poly-L-lysine was defined as 100 % adhesion. The results show that in the presence of 4F2 or NCAM, adhesion to laminin is enhanced. In the presence of both, there was no difference between adhesion with NCAM or 4F2 alone. The results shown are the mean and SEM of 3 independent experiments.

6.2 DISCUSSION

A physical association between CD98 and $\beta 1$ has been previously demonstrated (Chapter 5). This association appeared to be important in regulating signalling through integrins. As well as a link with $\beta 1$, Kakugwa et al, (2003) reported that CD98 stimulation could activate CEA-CAM-1 mediated cell adhesion via protein kinase C δ (PKC δ) activation. This study indicated that CD98 could regulate different types of adhesion molecules therefore it was decided to investigate this theory and find out whether there were any similar molecules to CD98 in terms of integrin regulation. In 1997, Beggs et al, showed that the neural cell adhesion molecule (NCAM) interacted with both p125^{fak} and p59^{lyn} non-receptor tyrosine kinases. The involvement of these two molecules in NCAM function suggested a convergence of NCAM and integrin dependent adhesion pathways. Therefore having studied the literature further, NCAM appeared to be an interesting molecule to investigate.

NCAM, the neural cell adhesion molecule is a member of the immunoglobulin (Ig) superfamily. It plays a pivotal role in the promotion of neural development and neural regeneration. NCAM exhibits a high structural diversity and a complex expression pattern. This is due to alternative splicing, developmental regulation and post translational processing. Three isoforms of NCAM are formed by alternative splicing of a single gene, these are the transmembrane forms, NCAM-140 and NCAM-180 and the glycosylphosphatidylinositol-linked (GPI) isoform, NCAM -120.

In order to investigate NCAM and its potential role in integrin signalling, the SCLC cell lines (Small cell lung cancer), H345, H69 and H510 were used. SCLC accounts for 20-25% of human cancers (Guo et al., 2000). Small cell lung carcinoma is a highly malignant tumour comprising cells with endocrine characteristics (Scheidegger et al., 1994). These endocrine characteristics include the production of dopa decarboxylase and neuron-specific enolase, secretion of various neuropeptides and the expression of neuronal surface markers such as the neural cell adhesion molecule and HNK-1 (Guo et al., 2000). SCLC cells generally grow as floating spheroid aggregates and are typically

anchorage independent (Doyle et al., 1990). The aggregates form through the process of cell-cell adhesion, which is mediated by E-cadherin and NCAM (Guo et al., 2000). Unlike the majority of other tumour cells, the expression of NCAM is upregulated rather than downregulated during tumour progression in SCLC (Cavallaro and Christofori, 2004). This makes the SCLC cell lines interesting tools to investigate NCAM expression and function.

Whilst confirming that endogenous NCAM was present in the SCLC cell lines obtained, it was discovered that the NCAM isoform expressed differed in terms of the SCLC cell line. H345 only expressed NCAM-180 whereas H69 expressed both transmembrane isoforms NCAM-140 and NCAM-180. This may be explained by the finding that in various tumour types, NCAM expression shifts from the adult GPI-linked 120 kDa isoform to the embryonic 140-180 kDa transmembrane isoforms (Cavallaro and Christofori, 2001; Christofori, 2003). This isoform switch and its significance is as yet unknown however it may be based on differential polysialylation of the various NCAM isoforms (Muhlenhott et al., 1996). Moolenaar et al., (1992) discovered that the SCLC cell line H69 cells expressed the two major isoforms of NCAM, 140 kDa and 180 kDa. They found no evidence for the 120 kDa isoform as no mRNA containing exon 15 was present, encoding the glycosyl-phosphatidylinositol-linked (GPI-linked) NCAM isoform.

NCAM has been shown to associate with a variety of different molecules. Beggs et al., (1997), showed that two non-receptor tyrosine kinases, p59^{l^yn} and p125^{fak} interact with the 140kDa isoform of NCAM. When cell surface NCAM 140 was ligated using antibodies or stimulated with NCAM-Fc (NCAM fusion protein consisting of the entire NCAM extracellular domain fused to the Fc region of human IgG), there was a transient increase in the tyrosine phosphorylation of both p125^{fak} and p59^{l^yn}. They suggested that activation of these non receptor tyrosine kinases is a proximal event in the NCAM signal transduction pathway.

Adaptor proteins such as FRS2 and Shc and the downstream effector PLC γ were found to associate with NCAM as well as FGFR-4. These associations suggest a role for

the cell adhesion molecule NCAM in mediating many different cellular functions via various signalling pathways.

To investigate whether the molecules CD98 and NCAM colocalise, dual label immunofluorescence studies were carried out. Using the SCLC cell line H69 which highly expresses CD98 and NCAM, I have demonstrated that these molecules colocalise within the membrane. This finding was further confirmed by investigating another molecule CD71. CD71, the transferrin receptor which is expressed on SCLC cells was used as a negative control. No colocalisation between NCAM and CD71 was seen indicating that the colocalisation between CD98 and NCAM was not a consequence of non-specific binding or close proximity in the membrane but suggests these molecules may interact. To examine this further, membranes were solubilised and a molecular association was examined by immunoprecipitation. These experiments confirmed that CD98 was associated with NCAM and was immunoprecipitated with NCAM by NCAM-specific antibodies which recognised both 140 kDa and 180 kDa isoforms. This suggested that the physical association observed between NCAM and CD98 was a real event and not just a consequence of the molecules being in the same place on the membrane. This result was confirmed again using CD71, which did not immunoprecipitate with NCAM. The reverse experiment where 4F2 was used to precipitate CD98 from the cell membrane showed in addition that 4F2 pulled down immunoreactive bands for NCAM 140 and 180 in H69 cells.

The suggested link between NCAM and integrin signalling prompted an investigation into a possible association between NCAM and $\beta 1$ integrin. This was again carried out using confocal microscopy and co-immunoprecipitation experiments. Partial colocalisation between NCAM and $\beta 1$ was seen and this was confirmed by the co-immunoprecipitation of both CD98 and $\beta 1$. The association between NCAM and $\beta 1$ has been investigated by Cavallaro et al, in 2001. They found that neutralizing antibodies to $\beta 1$ integrin repressed neurite outgrowth and matrix adhesion. They also showed that expression of the different NCAM-isoforms by transient transfection in NCAM^{-/-} tumour cells restored neurite outgrowth and cell adhesion, which in turn could be repressed by antibodies against $\beta 1$ integrin. These findings suggested that $\beta 1$ integrin is directly

activated by NCAM-mediated signalling. However this group was unable to show co-immunoprecipitation between $\beta 1$ and NCAM in NCAM^{+/+} tumour cells. Although uncertain, the association between NCAM and CD98 and the link between CD98 and $\beta 1$ nevertheless suggest that $\beta 1$ may be involved in NCAM-mediated signalling and may be part of an NCAM-signalling complex.

Having proposed the idea of a signalling complex, consisting of NCAM and CD98, it seemed sensible to investigate whether this association had any functional relevance. NCAM plays a major role in neural development and neural regeneration but also appears to have a potential role in the early metastatic characteristics of tumours. In chapter 4, I demonstrated that overexpression of CD98 induced anchorage independence, a key feature of tumorigenesis, and stimulated PI3-Kinase activation and its downstream effector AKT, an important property of oncogenes. In 2003, Ditlevsen et al., showed that activation of PI3-Kinase was necessary for NCAM-mediated neurite outgrowth and survival. They suggested that this may be a consequence of either FGFR-mediated NCAM activation, induced by recruitment of multiple docking proteins such as Gab1 generating binding sites for the SH2-domain of the p85 subunit of PI3-Kinase, (Ong et al., 2001) or the involvement of FAK where the p85 subunit binds to the phosphorylated FAK (phosphorylated upon binding to the NCAM-fyn complex, (Beggs et al., 1997)), activating PI3-Kinase, (Chen et al., 1996). Studies with human tumour biopsies and mouse tumour models have also revealed that NCAM plays an important role in the progression to tumour malignancy, (Cavallaro and Christofori, 2004). Therefore NCAM and CD98 appear to share many functional properties. Adhesion and colony assays were performed to investigate whether the expression and activation of both these molecules has any effect on these functional processes.

The results from both the colony assay and adhesion assays suggest that NCAM may play a role in anchorage independence and cell adhesion. NCAM activation by NCAM-Fc increased colony formation and adhesion to laminin. A similar though more pronounced effect was shown by crosslinking CD98. Co-activation of NCAM in addition to CD98 activation did not produce any additive effects on adhesion or colony formation, however further dose ranging studies are required to more fully answer this

question. Polysialylation of NCAM is very important in understanding the functional effects of NCAM. A unique structural feature of NCAM is the presence of polysialic acid, a developmentally regulated carbohydrate composed of a linear homopolymer of α (2,8)-linked sialic acid residues (Finne et al., 1983). This carbohydrate is added to specific N-glycan attachment sites in the fifth immunoglobulinlike domain of NCAM (Muhlenhoff et al., 1998). Polysialylation (PSA) of NCAM is a unique post translational modification that appears during vertebrate development to carry out a variety of biological functions, (Rutishauser and Landmesser, 1996). It is thought to facilitate cell migration, axon pathfinding and synaptogenesis, (Fujimoto et al., 2001). PSA-NCAM appears to increase metastatic potential and has been correlated with tumour progression and poor prognosis, (Seidenfaden et al., 2003). Polysialylation of NCAM has therefore been suggested as a possible mechanism which contributes to the lack of cell adhesion in human lung cancers and neuroblastomas. The polysialylation of NCAM therefore may explain my results which showed that NCAM enhanced cloning efficiency. The upregulation of NCAM expression seen in SCLC is also accompanied by NCAM polysialylation (Bruses and Rutishauser, 2001). The results from the adhesion assays showed a minor increase in adhesion to laminin after stimulation with NCAM-Fc. However more confusing was the lack of cell adhesion when plated on NCAM Fc. The lack of effect of NCAM-Fc could simply be a consequence of the concentration of NCAM-Fc used to coat the plates being insufficient to cause any effect. It could also be due to the NCAM-Fc being washed off during the process of the assay. Another possible explanation for the lack of effect of NCAM-Fc could be due to the endogenous NCAM in SCLC cells being bound to other NCAM molecules on neighbouring cells, so therefore may not be available to be stimulated by NCAM-Fc.

Unfortunately due to time and resource constraints I was unable to research these functions further to obtain a greater understanding in NCAM function. However if I had had the time, I would have carried out both the colony and adhesion assays again but using suboptimal level of both 4F2 and NCAM-Fc. I would also remove the PSA from the SCLC cells by treating the cells with endoneuraminidase, as carried out by Scheidegger et al., (1994) in which the effects of PSA were investigated in terms of its

role in tumour cell growth and differentiation. This study showed that expression of PSA affects NCAM-dependent signalling involved in tumourigenesis. Removal of this carbohydrate may therefore be useful in obtaining a more conclusive answer. PI3-kinase assays in the presence of both NCAM and CD98 would be useful experiments to elucidate whether PI3-Kinase activation is involved in NCAM-mediated signalling in SCLC as well as colony assays using cells stimulated with NCAM-Fc and 4F2 in the presence or absence of a PI3kinase inhibitor. Another interesting assay to carry out would be a proliferation assay, Pardoe et al., suggested a role for FGFR's in inducing increased proliferation in SCLC, therefore CD98 and NCAM may also be involved in this process. If it was possible I would have transiently transfected CHO K1 cells with NCAM and CD98 and investigated whether the Ras-MAPK pathway was activated via SDS-PAGE analysis, as the Ras pathway has been suggested as a method of signal transduction for modulation of expression of genes required for neuronal growth and survival, (Schmid et al., 1999).

This chapter has therefore demonstrated an association between CD98 and NCAM and NCAM and $\beta 1$,. The associations between these molecules may form a signalling complex and crosstalk between the integrin and growth factors may modulate the signals produced, influencing the surrounding cellular environment, leading to transformation.

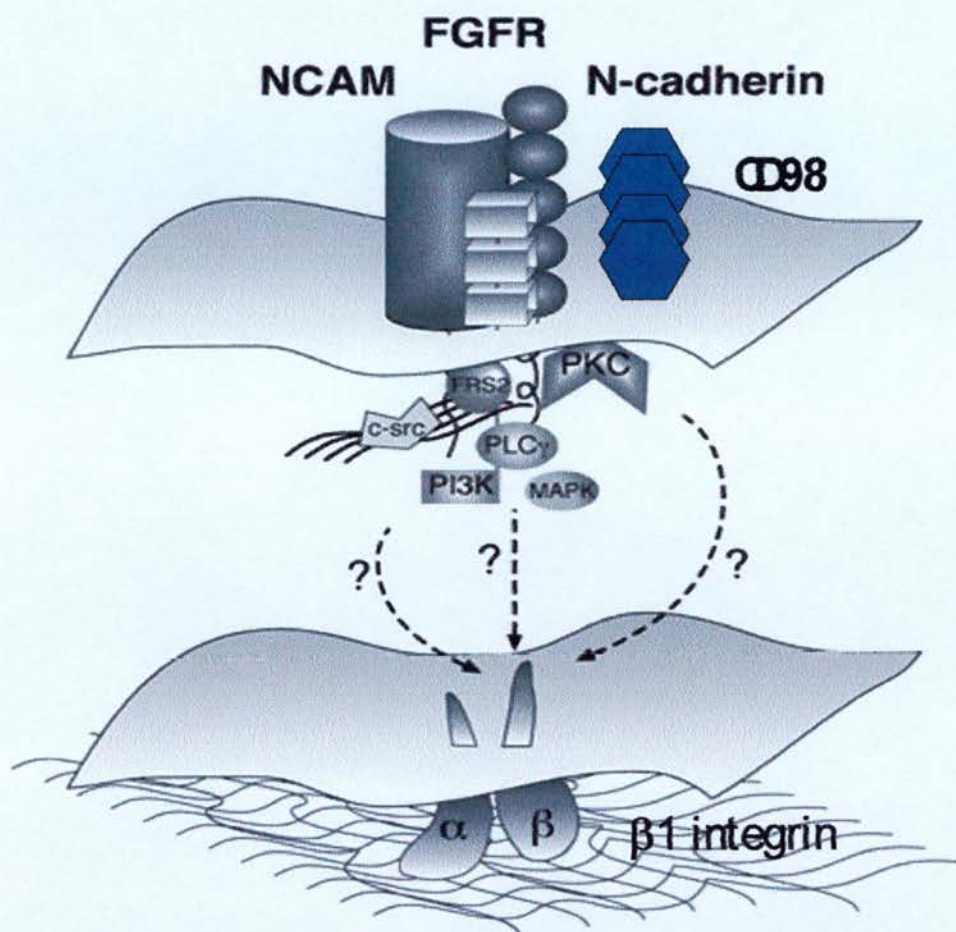


Figure 6.2. Schematic representation of proposed NCAM/CD98/ FGFR/N-cadherin signalling complex.

An signalling complex consisting of NCAM, N-cadherin, FGFR and CD98 forms, mediating the signal transduction pathways. The crosstalk between the growth factor receptor and integrins modulate the signals produced, influencing the surrounding cellular environment leading to transformation. The dashed lines represent the as of yet poorly understood signalling pathways linking the NCAM complex to integrins. This diagram is adapted from Cavallaro and Christofori, 2004.

CHAPTER 7

FINAL CONCLUSIONS AND FUTURE DIRECTIONS

Adhesion to the extracellular matrix is a pivotal requirement for survival of normal cells. This relationship between the cell and the extracellular matrix is regulated by components of the ECM or cell surface ligands and governed by members of a family of cell-surface adhesion receptors known as integrins. In addition to their roles as adhesion receptors, integrins also play a role in signalling. Much is known about the extracellular interactions between integrins and their ligands however, significantly less is known about the biochemical pathways that the integrins regulate and the cellular functions which are thereby controlled (Clark and Brugge, 1995).

CD98 is a 125 kDa heterodimeric transmembrane glycoprotein amino acid transporter. Recent studies have indicated that CD98 is also a regulator of integrin activation (Fenczik et al., 1997; Zent et al., 2000; Warren et al., 2000). These studies suggested that CD98 is involved in the regulation of ligand binding activity of integrins. There is also increasing evidence that CD98 has oncogenic potential. Most tissues and tumour cells have been shown to have a high expression of the CD98 heavy chain (Parmacek et al., 1989; Nakamura et al., 1999) and increased CD98 expression has been shown to correlate with the development, progression and metastatic potential of tumours (Esteban et al., 1990; Garber et al., 2001; Yoon et al., 2003). The central aim of my thesis was to investigate the diverse effects of CD98 and to elucidate which part of the CD98 molecule was important for these processes. This was carried out using a series of CD98 chimeras.

Endogenous levels of CD98 do not result in a malignant phenotype. However the results of this study suggest that constitutively high levels of CD98 expression result in both anchorage and serum independent growth. This oncogenic activity is dependent on PI3-Kinase activation. This finding is in agreement with Moore et al., 1998, in which they found that PI3 kinase acting through AKT promoted

anchorage independence. Rintoul et al., (2002), also showed that when CD98 was crosslinked PI3-Kinase activation was stimulated. The study of CD98 chimeric constructs suggest that the transmembrane domain is required for the mediation of oncogenic transformation. In addition the transmembrane domain of the CD98 heavy chain was sufficient to promote extensive focal adhesion complexes and to induce these phenotypic changes. The chimeras which had the transmembrane domain of CD98 heavy chain replaced with that of CD69 lost the capacity to stimulate PI3-Kinase and promote anchorage independence. Studies have shown that the mutation of extracellular cysteines C109S and C330S of CD98HC disrupt the covalent association with the light chain but do not impair interactions or effects on integrins (Fenczik et al., 2001). The extracellular domain of CD98 when exchanged with that of CD69 however did not appear to affect these processes. This implies that the covalent association of the CD98 heavy chain with the light chain is not required for the functional regulation of integrins. More specifically, studies with the chimeras, 98TM and the mutants Δ 4F2 and D5, suggested that the amino acids 82-86 were critical for these processes. In addition to this I found that CD98 heavy chain over-expression had no effect on the actin cytoskeleton and the microtubule network despite being able to promote transformation and PI3-Kinase activity.

Rintoul et al., (2002), demonstrated that CD98 and β 1 colocalised and co-immunoprecipitated in SCLC cells. CHOK1 cells stably transfected with CD98, CD69 or the chimeras, showed colocalisation between CD98 and β 1 in the CD98 stables but not in the negative control CD69 stables. The colocalisation of CD98 and β 1 appeared to be dependent on the presence of the transmembrane domain of CD98. The chimera, 4F269 which had the transmembrane domain of CD98 missing and replaced with that of CD69 did not show colocalisation with β 1. Deconvolution and surface rendering confirmed these results. Co-immunoprecipitation experiments corroborated these results indicating that the amino acids 82-86 of CD98 were also important for integrin association as well as PI3-Kinase activation and anchorage independent growth. However there were some exceptions. The chimera 69TM co-immunoprecipitated with β 1 but did not show colocalisation. This finding was further confused by the truncation

mutant D5. The D5 mutant, which lacked the amino acids 1-86, but did have an intact extracellular domain of CD98, did not show colocalisation with the $\beta 1$ integrin. This may be a consequence of these four amino acids being absent. These results implied that the $\beta 1$ molecule may bind to either the extracellular domain of CD98 or that the transmembrane domain may induce a change in the conformation of the extracellular domain promoting CD98 and $\beta 1$ association.

Kakugwa et al, (2003) reported that CD98 stimulation could activate CEA-CAM-1 mediated cell adhesion via protein kinase C δ (PKC δ) activation. This study suggested that CD98 may regulate different types of adhesion molecules. NCAM appeared to be a potential candidate. I found that the NCAM isoform expressed differed in terms of the SCLC cell line. H345 only expressed NCAM 180 whereas H69 expressed both transmembrane isoforms NCAM-140 and NCAM-180. This may be explained by the isoform switch seen in various tumour types (Cavallaro and Christofori, 2001; Christofori, 2003). A possible link between CD98 and NCAM, NCAM and $\beta 1$ and was demonstrated using colocalisation and co-immunoprecipitation studies. Cavallaro et al., (2001), suggested that NCAM modulates neurite outgrowth and matrix adhesion by assembling a signalling complex consisting of FGFR-4 and various other signalling molecules such as N-cadherin, PLC γ and cortactin. Unfortunately the investigation into the functional relevance of an association between CD98 and NCAM was not completed due to time and resource constraints. The results which were obtained suggest a role for NCAM in similar functional processes as CD98, such as anchorage independence and adhesion. However the post-translational modification polysialylation of NCAM appears to be very important in understanding the functions of NCAM.

It is important to be aware that these studies have been carried out in CHOK1 cells which overexpress the CD98 molecule. Crosslinking CD98 using the monoclonal antibody 4F2 also clusters the CD98 molecules together causing overexpression of CD98. This overexpression of protein may therefore affect normal cellular events. To combat this problem, methods such as siRNA and dominant negative protein expression

could be used and compared against control cells. This would help further confirm the results obtained in this investigation.

Overall the results obtained in this thesis have demonstrated that the heavy chain of CD98 is an oncogene which promotes transformation when overexpressed in CHOK1 cells. This oncogenic activity is dependent on PI3-Kinase activation and the level of CD98 cell surface expression. Using the CD98 chimeras, it has been established that the transmembrane domain of CD98, specifically the amino acid residues 82-86, is necessary and sufficient for integrin association, PI3-kinase activation, anchorage independence and the induction of phenotypical changes such as extensive focal adhesion formation.

Evidence has shown that CD98 plays an important role in inflammation and viral diseases in addition to the role it plays in cancer via its effects on cellular activation and integrin regulation. Abnormalities in the function and regulation of ECM interactions have recently been implicated in the etiology of various inflammatory disorders such as inflammatory bowel disease. Kucharzik et al., (2005) found in Caco2- BBE monolayers and colonic tissues that the expression of CD98 was upregulated by the proinflammatory cytokine INF γ . This study found evidence that the activation of CD98 aggravates intestinal inflammation. All this mounting evidence suggests that CD98 is an important molecule to study not only as a mediator of transformation but also as a potential therapy target for many inflammatory diseases.

As approximately 90% of all cancer deaths arise from metastasis (Cavallaro and Christofori, 2004), understanding the molecular mechanisms involved in tumour progression, invasion and metastasis, may provide unique targets for cancer therapy. Recent advances in research have indicated that changes in cell adhesion play critical roles in tumour progression. The association between CD98 and the cell adhesion molecule NCAM, as well as the possible link between β 1 and FGFR-2, may provide a method of modulating the signals produced from the crosstalk between integrins and growth factors, by forming a signaling complex. This complex may therefore influence the signals being produced leading to the process of oncogenic transformation.

The work in this thesis has provided the groundwork for this important area of study and has identified key targets for further research.

Table 5 provides a summary of all the findings made in this thesis with regards to the CD98 chimeras.

Table 5

<u>Chimera</u>	<u>Anchorage Independence</u>	<u>Colocalisation with $\beta 1$ integrin</u>	<u>Co-immunoprecipitation with $\beta 1$</u>	<u>PI3-kinase activation</u>
Full length CD98	Y	Y	Y	Y
Full lengthh CD69	N	N	N	N
4F269	N	N	N	N
4F2TM	Y	Y	Y	Y
69CYTO	Y	Y	Y	Y
69TM	N	N	Y	N
98EX	N	Y	Y	N
98TM	Y	Y	Y	Y
Δ 4F2	Y	Y	Y	Y
D5	N	N	?	N

Table 5 Summary of the CD98 Chimera effects

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